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(54) Title: ANTI-HUMAN MILK FAT GLOBULE HUMANISED ANTIBODIES

(57) Abstract

Humanised antibody molecules (HAMs) are described having specificity for human milk fat globule and having an antigen binding site wherein at least one of the complementarity determining regions (CDRs) of the variable domains is derived from the mouse monoclonal antibody CTMO1 and the remaining immunoglobulin-derived parts of the HAM are derived from a human immunoglobulin. The HAMs may be chimeric humanised antibodies or CDR-grafted humanised antibodies and are preferably produced by recombinant DNA techniques. The HAMs may be conjugated to an effector or reporter molecule, particularly methyltrithio anti-tumour agents, and are useful for *in vivo* diagnosis and therapy.

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ANTI-HUMAN MILK FAT GLOBULE HUMANISED ANTIBODIES

FIELD OF THE INVENTION

The present invention relates to humanised antibody molecules (HAMs) having specificity for human milk fat globule (HMFG) and to processes for their production using recombinant DNA technology.

BACKGROUND TO THE INVENTION

The term "humanised antibody molecule" (HAM) is used to describe a molecule having an antigen binding site derived from an immunoglobulin from a non-human species, the remaining immunoglobulin-derived parts of the molecule being derived from a human immunoglobulin. The antigen binding site may comprise: either a complete variable domain from the non-human immunoglobulin fused onto one or more human constant domains; or one or more of the complementarity determining regions (CDRs) grafted onto appropriate human framework regions in the variable domain. The abbreviation "MAb" is used to indicate a monoclonal antibody.

In the description, reference is made to publications by number. These numbers are placed in square brackets [ ]. The publications are listed in numerical order at the end of the description.

Natural immunoglobulins have been known for many years, as have the various fragments thereof, such as the Fab, Fab' $(Fab')_2$ and Fc fragments, which can be derived by enzymatic cleavage. Natural immunoglobulins comprise a generally Y-shaped molecule having an antigen-binding site towards the outer end of each arm. The remainder of the structure, and particularly the stem of the Y, mediates the effector functions associated with immunoglobulins.

Natural immunoglobulins have been used in assay, diagnosis and, to a more limited extent, therapy. However, such uses, especially in therapy, have been hindered by the polyclonal nature of natural

immunoglobulins. A significant step towards the realisation of the potential of immunoglobulins as therapeutic agents was the discovery of procedures for the production of monoclonal antibodies of defined specificity [1]. However, most MAbs are produced by hybridomas which are fusions of rodent spleen cells with rodent myeloma cells. The resultant MAbs are therefore essentially rodent proteins. There are few reports of the production of human MAbs.

Since most available MAbs are of rodent origin, they are naturally antigenic in humans and thus can give rise to an undesirable immune response termed the HAMA (Human Anti-Mouse Antibody) response. Therefore, the use of rodent MAbs as therapeutic agents in humans is inherently limited by the fact that the human subject will mount an immunological response to the MAb and will either remove it entirely or at least reduce its effectiveness.

Therefore proposals have been made for making non-human MAbs less antigenic in humans. Such techniques can be generically termed "humanisation" techniques. These techniques generally involve the use of recombinant DNA technology to manipulate DNA sequences encoding the polypeptide chains of the antibody molecule.

Early methods for humanising MAbs related to production of chimeric antibodies in which an antigen binding site comprising the complete variable domains of one antibody are fused to constant domains derived from a second antibody. Methods for carrying out such chimerisation procedures are described in EP-A-0 120 694 (Celltech Limited), EP-A-0 125 023 (Genentech Inc.), EP-A-0 171 496 (Res. Dev. Corp. Japan), EP-A-0173494 (Stanford University) and EP-A-0 194 276 (Celltech Limited).

EP-A-0 194 276 discloses a process for preparing an antibody molecule having the variable domains from a mouse MAb and the constant domains from a human immunoglobulin. It also describes the production of an antibody molecule comprising the variable domains of a mouse MAb, the CH1 and CL domains of a human immunoglobulin

and a non-immunoglobulin-derived protein in place of the Fc portion of the human immunoglobulin.

Subsequently, a number of further patent applications have been published relating to chimeric antibodies, including tumour specific chimeric antibodies. Among these applications are WO-A-87/02671 (Int. Gen. Eng. Inc.), EP-A-0 256 654 (Centocor), EP-A-0 266 663 (Int. Gen. Eng. Inc. & Oncogen), WO-A-89/00999 (Int. Gen. Eng. Inc.) and EP-A-0 332 424 (Hybritech Inc.).

Such humanised chimeric antibodies, however, still contain a significant proportion of non-human amino acid sequence, i.e. the complete variable domains. Thus, such humanised antibodies may elicit some HAMA response, particularly if administered over a prolonged period [2].

In an alternative approach, described in EP-A-0 239 400 (Winter), the complementarity determining regions (CDRs) of a mouse MAb have been grafted onto the framework regions of the variable domains of a human immunoglobulin by site directed mutagenesis using long oligonucleotides. Such CDR-grafted humanised antibodies are less likely to give rise to a HAMA response than humanised chimeric antibodies in view of the lower proportion of non-human amino acid sequence which they contain. There are three CDRs (CDR1, CDR2 and CDR3) in each of the heavy and light chain variable domains.

The earliest work on CDR-grafted humanised MAbs was carried out on a MAb recognising the synthetic antigen NP or NIP. However, subsequently, examples in which a mouse MAb recognising lysozyme and a rat MAb recognising an antigen on human T cells respectively were humanised have been described [3, 4]. The preparation of the CDR-grafted antibody to the antigen on human T cells is also described in WO-A-89/07452 (Medical Research Council). Recently the preparation of a humanised CDR-grafted antibody that binds to the interleukin 2 receptor has been described [5]. Further examples of humanised CDR-grated antibodies having specificity for anti-viral [6, 7],

anti-tumour [8] and anti-T cell [9 and EP-A-0 403 156] antigens have been described more recently.

Our copending International Patent Specification No. WO-A-91/09967 relates to the CDR grafting of antibodies in general.

It has been widely suggested that immunoglobulins, and in particular MAbs, could potentially be very useful in the diagnosis and treatment of cancer [10, 11]. There has therefore been much activity in trying to produce immunoglobulins or MAbs directed against tumour-specific antigens. So far, over one hundred MAbs directed against a variety of human carcinomas have been used in various aspects of tumour diagnosis or treatment [12].

There have been a number of papers published concerning the production of chimeric monoclonal antibodies recognising cell surface antigens. For instance, genetically engineered murine/human chimeric antibodies which retain specificity for tumour-associated antigens have been described [13 and WO-A-89/01783]. Also, a recombinant murine/human chimeric monoclonal antibody specific for common acute lymphocytic leukaemia antigen has been described [14].

SUMMARY OF THE INVENTION

We have now prepared humanised antibodies to human milk fat globule (HMFG) derived from the anti-HMFG mouse MAb CTMO1 [15].

According to the present invention, there is provided a humanised antibody molecule (HAM) having specificity for human milk fat globule (HMFG) and having an antigen binding site wherein at least one of the complementarity determining regions (CDRs) of the variable domain is derived from the mouse monoclonal antibody CTMO1 (CTMO1 MAb) and the remaining immunoglobulin-derived parts of the HAM are derived from a human immunoglobulin or an analogue thereof.

The HAM may comprise a chimeric humanised antibody or a CDR-grafted humanised antibody. When the HAM comprises a CDR-grafted humanised antibody, each heavy or light chain variable domain may

comprise only one or two CTMO1-derived CDRs. Preferably, however, all three heavy and light chain CDRs are derived from CTMO1.

The CTMO1 MAb is a mouse MAb of the type IgG1-kappa raised against the membrane-associated antigen of HMFG and has been extensively studied [15]. The CTMO1 MAb has been shown to recognise breast, ovarian and non-small cell lung cancers. It has been shown to internalise rapidly into target cells. Conjugates of CTMO1 and calichaemicin display highly specific cytotoxicity against appropriate cell lines, (see USP 5053394).

High levels of the antigen recognised by the CTMO1 MAb have been detected circulating in the blood of patients suffering from breast cancer. This may have a deleterious effect on pharmacokinetics and tumour localisation *in vivo*. However, circulating antigen levels in the blood of patients suffering from ovarian cancer are lower than those in breast cancer patients. It is therefore believed that the HAM of the present invention will be of particular use in the treatment of ovarian cancer.

It is believed that the CTMO1 MAb recognises the polymorphic epithelial mucin (PEM) of HMFG. Thus, preferably, the present invention provides a HAM which recognises the PEM of HMFG.

Surprisingly, it has been found that humanising the CTM01 MAb does not substantially adversely affect its binding activity or internalisation, and can create, particularly by CDR grafting, a HAM which has better binding and internalisation characteristics than the murine antibody (see Table 1 hereinafter). This produces a HAM which is of use in both therapy and diagnosis of certain human carcinomas, for example carcinomas of ovary, breast, uterus and lung.

Preferably, the HAM of the present invention is produced by recombinant DNA technology.

The HAM of the present invention may comprise: a complete antibody molecule, having full length heavy and light chains; a fragment thereof,

such as an Fab, Fab', $(Fab')_2$ or Fv fragment; a single chain antibody fragment, e.g. a single chain Fv; a light chain or heavy chain monomer or dimer; or a fragment or analogue of any of these or any other molecule with the same specificitiy as the CTMO1 MAb.

The remaining non-CTMO1 immunoglobulin-derived parts of the HAM may be derived from a suitable human immunoglobulin. For instance, when the HAM is a CDR-grafted HAM, appropriate variable region framework sequences may be used having regard to the class or type of the CTMO1 donor antibody from which the antigen binding regions are derived. Preferably, the type of human framework used is of the same or similar class or type as the donor antibody (CTMO1 is IgG1-kappa). Advantageously, the framework is chosen to maximise or optimise homology with the donor antibody sequence, particularly at positions spatially close to or adjacent the CDRs. Examples of human frameworks which may be used to construct CDR-grafted HAMs are LAY, POM, TUR, TEI, KOL, NEWM, REI and EU [16]. KOL and NEWM are suitable for heavy chain construction. REI is suitable for light chain construction. EU is particularly suitable for both heavy chain and light chain construction. Preferably, the EU framework is used as the human framework for both heavy and light chain variable domains in view of its high level of homology with the CTMO1 MAb.

The light or heavy chain variable regions of the HAM may be fused to human light or heavy chain constant domains as appropriate, (the term "heavy chain constant domains" as used herein are to be understood to include hinge regions unless specified otherwise). The human constant domains of the HAM, where present, may be selected having regard to the proposed function of the antibody, in particular the effector functions which may be required. For example, the heavy chain constant domains fused to the heavy chain variable region may be human IgA, IgG or IgM domains. Preferably human IgG domains are used. IgG1 and IgG3 isotype domains may be used when the HAM is intended for therapeutic purposes and antibody effector functions are required. Alternatively, IgG2 and IgG4 isotype domains may be used when the HAM is intended for purposes for which antibody effector functions are not required, e.g. for imaging, diagnostic

or cytotoxic targeting purposes. Light chain human constant domains which may be fused to the light chain variable region include human Lambda or, especially, human Kappa chains.

Analogues of human constant domains may alternatively be advantageously used. These include those constant domains containing one or more additional amino acids than the corresponding human domain, or those constant domains wherein one or more existing amino acids of the corresponding human domain has been deleted or altered. Such domains may be obtained, for example, by oligonucleotide directed mutagenesis. In the present invention, a particularly useful analogue of a heavy chain constant domain is an IgG4 constant domain in which a serine residue at position 241 of the corresponding naturally occuring human domain has been altered to a proline residue.

The remainder of the HAM need not comprise only protein sequences from human immunoglobulins. For instance, a gene may be constructed in which a DNA sequence encoding part of a human immunoglobulin chain is fused to a DNA sequence encoding the amino acid sequences of a polypeptide effector or reporter molecule.

According to a second aspect of the present invention, there is provided a process for producing the HAM of the first aspect of the invention, which process comprises:

(a) producing in an expression vector an operon having a DNA sequence which encodes an antibody heavy or light chain comprising a variable domain wherein at least one of the CDRs of the variable domain is derived from the CTMO1 MAb and the remaining immunoglobulin-derived parts of the antibody chain are derived from a human immunoglobulin;

(b) producing in an expression vector an operon having a DNA sequence which encodes a complementary antibody light or heavy chain comprising a variable domain wherein at least one of the CDRs of the variable domain is derived from the

CTMO1 MAb and the remaining immunoglobulin-derived parts of the antibody chain are derived from a human immunoglobulin;

(c) transfecting a host cell with both operons; and

(d) culturing the transfected cell line to produce the HAM.

The cell line may be transfected with two vectors, the first vector containing the operon encoding the light chain-derived polypeptide and the second vector containing the operon encoding the heavy chain-derived polypeptide. Preferably, the vectors are identical except in so far as the coding sequences and selectable markers are concerned so as to ensure as far as possible that each polypeptide chain is equally expressed.

Alternatively, a single vector may be used, the vector including the operons encoding both light chain- and heavy chain-derived polypeptides.

In further aspects, the invention also includes DNA sequences coding for the heavy and light chains of the HAM of the present invention, cloning and expression vectors containing these DNA sequences, host cells transformed with these DNA sequences and processes for producing the heavy or light chains and antibody molecules comprising expressing these DNA sequences in a transformed host cell.

The general methods by which the vectors may be constructed, transfection methods and culture methods are well known per se [17, 18].

The DNA sequences which encode the CTMO1 heavy and light chain variable domain amino acid sequences (and the corresponding deduced amino acid sequences) are given hereinafter in the sequence listing as Sequence ID No. 1 and Sequence ID No. 2 respectively.

DNA coding for human immunoglobulin sequences may be obtained in any appropriate way. For example, amino acid sequences of preferred human acceptor frameworks, such as LAY, POM, KOL, REI, EU, TUR, TEI and NEWM, are widely available to workers in the art. Corresponding DNA sequences which code for these amino acid sequences may be inferred or deduced by reverse application of the genetic code. Similarly, the amino acid sequences of human constant region domains are well known and DNA sequences which code for them may be readily deduced.

The standard techniques of molecular biology may be used to prepare DNA sequences coding for CDR-grafted products. Desired DNA sequences may be synthesised completely or in part using oligonucleotide synthesis techniques. Site-directed mutagenesis and polymerase chain reaction (PCR) techniques may be used as appropriate. For example, oligonucleotide directed synthesis [19] may be used. Also, oligonucleotide directed mutagenesis of a pre-existing variable domain region [3, 4] may be used. Enzymatic filling-in of gapped oligonucleotides using T4 DNA polymerase [5] may be used.

Any suitable host cell/vector system may be used for expression of the DNA sequences coding for the chimeric or CDR-grafted heavy and light chains. Bacterial, e.g. *E. coli*, and other microbial systems may be used, in particular for expression of antibody fragments, e.g. Fv, Fab and Fab' fragments and single chain antibody fragments, e.g. single chain Fvs. Eucaryotic, e.g. mammalian host cell, expression systems may be used for production of larger chimeric or CDR-grafted antibody products, including complete antibody molecules. Suitable mammalian host cells include CHO cells and myeloma or hybridoma cell lines, for example NSO cells.

In a further aspect of the invention we provide a conjugate molecule comprising a HAM conjugated to an effector or reporter molecule. Thus for example the HAM of the present invention may have attached to it an effector molecule such as a cytotoxic or cytostatic agent, or a reporter group, for example an atom or molecule such as a radionuclide, or complexed radionuclide capable of being detected

while inside the human body. For instance, the HAM may have an organic group, such as a macrocycle, capable of binding a metal atom, or a toxin, such as ricin, or an anti-tumour agent as hereinafter defined, attached to it by a covalent bridging structure. Alternatively, the procedures of recombinant DNA technology may be used to produce a HAM in which the Fc fragment, CH2 or CH3 domain of a complete molecule has been replaced by or has attached thereto by peptide linkage a functional non-immunoglobulin protein, such as an enzyme or toxin molecule.

A particularly useful conjugate molecule according to this aspect of the invention is a HAM conjugated to a methyltrithio anti-tumour agent. Particular methyltrithio anti-tumour agents include the disulphide analogues of the α_1, α_2, α_3, α_4, β_1, β_2, γ_1, δ_1 and pseudoaglycone components of the LL-E33288 complex and derivatives thereof, as well as the disulphide analogues of 88M-1675, FR-900405, FR-900406, PD 114759, PD 115028, CL-1577A, CL-1577B, CL-1577D, CL-1577E and CL 1724 antitumour antibiotics and derivatives thereof.

The family of antibacterial and antitumor agents, known collectively as the LL-E33288 complex are described and claimed in U.S. Pat. No. 4,970,198 (1990) and are used to prepare the disulphide antitumour agents which are some of the starting materials for the conjugate molecules of the invention.

U.S. Patent 4,970,198 describes the LL-E33288 complex, the components thereof, namely, LL-E33288α_1^{Br}, LL-E33288α_1^{I}, LL-E33288α_2^{Br}, LL-E33288α_2^{I}, LL-E33288α_3^{Br}, LL-E33288α_3^{I}, LL-E33288α_4^{Br}, LL-E33288β_1^{Br}, LL-E33288β_1^{I}, LL-E33288β_2^{Br}, LL-E33288β_2^{I}, LL-E33288δ_1^{Br}, LL-E33288δ_1^{I}, LL-E33288δ_1^{I}, and methods for their production by aerobic fermentation utilizing a new strain of *Micromonospora echinospora ssp calichensis* or natural or derived mutants thereof. U.S. Pat. No. 4,970,198 also discloses proposed structures for some of the above-named components.

Additional members of the LL-E33288 complex (the calicheamicins) are described and claimed in U.S. Pat. No. 4,939,244 (1990) and are

likewise useful for preparing the conjugate molecules of the invention. This patent also describes the LL-E33288 bromo- and iodo-pseudoaglycones of the series, which have been prepared by chemical means. The patent also describes dihydro derivatives accessable from all the above-named antitumor antibiotics through sodium borohydride reduction of the ketone at C_{11} to a hydroxyl group.

Still other members of the LL-E33288 family of antitumour antibiotics are described and claimed in U.S. Patent 5,079,233 (1992), and also are useful for preparing additional conjugate molecules of the invention. This patent describes N-acyl derivatives of several members of the LL-E33288 complex which have been prepared by chemical means.

Other antibiotics are useful to prepare conjugate molecules of the invention, namely:

1) Esperamicin BBM-1675, [M. Konishi, et. al., J. Antibiotics, 38, 1605 (1985); M. Konishi, et. al., U.K. Patent Specification 2,141,425A, and U.S. Pat. No. 4,675,187].

2) Antitumour antibiotics, FR-900405 and FR-900406. [M. Iwami, et. al., J. Antibiotics, 38, 835 (1985), S. Kiyoto, et. al., J. Antibiotics, 38, 340 (1985)].

3) PD 114759 and PD 115028, [R.H. Bunge, et. al., J. Antibiotics, 37, 1566 (1984) U.S. Pat. No. 4,554,162 D.W. Fry et. al., Investigational New Drugs, 4, 3 (1986)].

4) Antibiotic complex CL-1577A, CL-1566B produced by *Streptomyces* asp. ATCC 39363. U.S. Pat. No. 4,539,203 (1985).

5) CL-1577D and CL-1577E Antibiotic antitumor compounds, U.S. Pat. No. 4,539,203.

6) CL-1724 Antibiotic compounds, U.S. Pat. No. 4,554,162.

7) Antitumour antibiotics BBM-1675-A3 and BBM-1675-A4, obtained by fermentation of actinomadura verrucosospora strains H964-92 (ATCC 39334) or AB27Y (ATCC 39638). U.S. Pat. No. 4,675,187.

8) N-acetyl-esperamicin A_1, A_2 and $A_{1\beta}$ derivatives with antimicrobial and antitumor activities. European Patent Specification 289,030.

All of the information regarding the LL-E33288 family of antitumor antibiotics, BBM-1675, FR-900405, FR-900406, PD 114759, PD 115028, CL-1577A, CL-1577B, CL-1577D, CL-1577E and CL-1724 contained in the above citations is incorporated herein by reference.

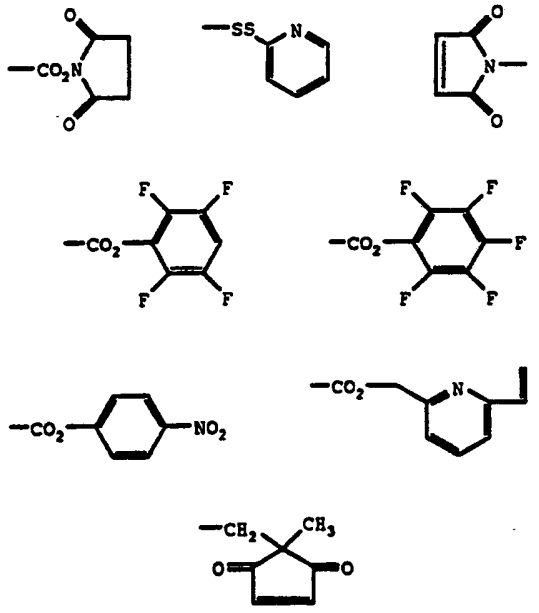
The α_1, α_2, α_3, α_4, β_1, β_2, γ_1, δ_1, and pseudoaglycone components of the LL-E33288 complex their dihydro and N-acyl counterparts, as well as the BBM-1675, FR-900405, FR-900406, PD 114759, PD 115028, CL-1577A, CL-1577B, CL-1577D, CL-1577E and CL-1724 antibiotics and their N-acyl counterparts, each contain a methyltrithio group in their structure. The methyltrithio moiety of the above-named antibiotics is subject to displacement by a variety of thiol-containing organic molecules resulting in the formation of a new class of anticancer and antibacterial agents as described in US Patent Application No. 07/825248 filed Jan. 24, 1992. Displacement of the methyltrithio unit of the antitumour antibiotics as depicted in Scheme I, below, can be used to introduce a spacer (Sp), the judicious choice of which enables the introduction of a HAM of the invention (hereinafter Hu:CT-M-01) into the compounds of the above-named patents and applications to form a conjugate molecule according to the invention.

Scheme 1

Ch_3SSS-W $\xrightarrow{\text{Q-Sp-SH}}$ Q-Sp-SS-W

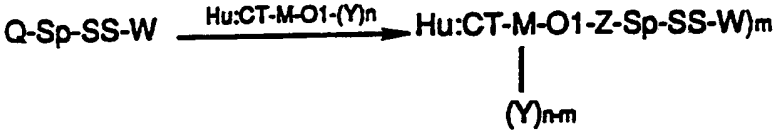
With reference to Scheme I CH_3-SSS-W is the antitumour antibiotic, Sp is a straight or branched-chain divalent or trivalent (C_1-C_{18}) radical,

divalent or trivalent aryl or heteroaryl radical, divalent or trivalent (C_3-C_{18}) cycloalkyl or heterocycloalkyl radical, divalent or trivalent aryl- or heteroaryl-alkyl (C_1-C_{18}) radical, divalent or trivalent cycloalkyl- or heterocycloalkyl-alkyl (C_1-C_{18}) radical, or divalent or trivalent (C_2-C_{18}) unsaturated alkyl radical, wherein if Sp is a trivalent radical, it can be additionally substituted by amino, alkylamino, arylamino, heteroarylamino, carboxyl, lower alkoxy, hydroxy, thiol or lower alkylthio groups; Q is, or can be subsequently converted to, halogen, amino, alkylamino, carboxyl, carboxaldehyde, hydroxy, thiol, a-haloacetyloxy, lower alkyldicarboxyl, -$CONHNH_2$, -$NHCONHNH_2$, -$NHCSNHNH_2$, -ONH_2, -CON_3,

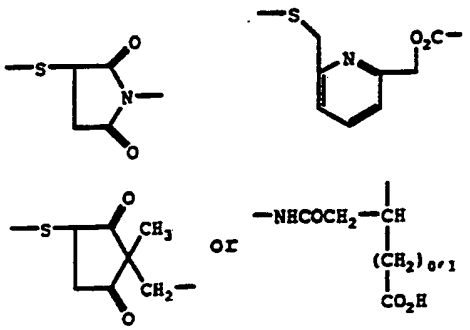


As long as the product from Scheme I contains at least one functional group which can be converted to, or is directly reactive with Hu:CT-M-01, targeted forms of the antitumor antibiotics of the above-named patents and applications can be generated, as shown in Scheme II below:

Scheme II



wherein Q, Sp, and W are as hereinbefore defined, Hu:CT-M-01 is a HAM, its fragments, or an analogue thereof; Y is a side-chain amino, carboxy, or thiol group of a protein, an aldehyde derived from carbohydrate residues, or an amidoalkylthio group; n is an integer of from 1 to 100; Z is formed from covalent reaction of the groups Q and Y directly or after subsequent reduction and Z is -CONH-, -CONHN=CH-, -CONHNHCH$_2$-, -NH-CONHN=CH-, -NHCONHNHCH$_2$-, -NHCSNHN=CH-, -NHCSNHNHCH$_2$-, -ON=CH-, -NH-, -NHCH$_2$-, -N=CH-, -CO$_2$-, -NHCH$_2$CO$_2$-, -SS-,



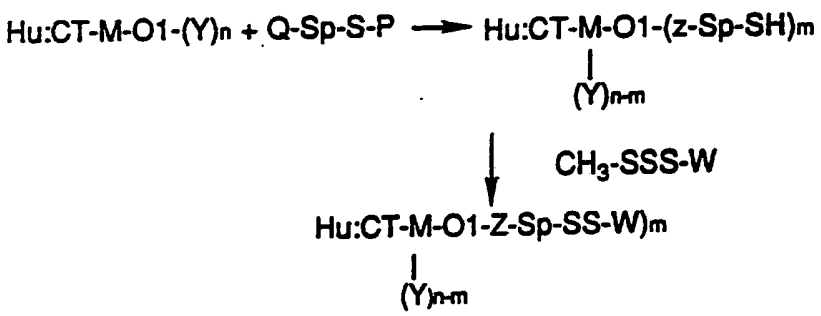
and *m* is 0.1 to 15.

As an example, and with reference to Scheme II, above, the 3-mercaptopropionic acid derivative of E-33288γ1^I (Q=CO$_2$H, Sp=-CH$_2$CH$_2$-), when converted to its activated hydroxysuccinimide form (Q=CO$_2$Su, Sp=-CH$_2$CH$_2$-) can be used to react with some of the e-amino groups of lysine residues (e.g., Y=-NH$_2$ wherein n=50-100 from available lysine residues), of Hu:CT-M-O1 at a pH between 7.0 and 9.5 in aqueous buffered solutions at temperatures between 4°C to 40°C to produceconjugate molecules of the invention with the antibiotics attached at random sites along the protein backbone (Z=-NHCO-, Sp=-CH$_2$CH$_2$, *m*=1-10). Only a fraction of the available lysine residues are substituted in this manner, since high loading is generally

not considered compatible with preserving the antibody immunoreactivity. The same randomly-substituted immunoconjugates can also be prepared from the 3-mercaptopropionic acid derivative using other carboxyl group activating agents such as a variety of carbodiimides, or the corresponding acyl azide. Alternatively, a 3-mercaptopropionyl hydrazide derivative of E-33288γ_1^I (Q=H_2NNHCO-, Sp=-CH_2CH_2-), when reacted with a periodate-oxidized antibody (Y=-CHO, N=1-15) as described in U.S. Pat. No. 4,671,958 at a pH between 4 and 7, in a buffered aqueous solution at a temperature of between 4°C and 40°C, reacts only at the aldehyde functionality (derived from cleavage of vic-diols of carbohydrate residues situated on the Fc portion of the antibodies) to generate Hu:CT-M-01 conjugates containing the drug substituted at specific sites along the backbone of the protein (Z=-CH=NNHCO-, Sp=-CH_2CH_2-, *m*=0.5-10). Other aldehyde-reactive groups as part of the drug construct are within our invention to generate the products of Scheme II. Such functional groups are preferably, though not limited to, those which react with aldehydes under acidic aqueous conditions. The reactivity of protein lysines under basic conditions is sufficiently great such that their amines compete with the products of Scheme II for available aldehydes of the monoclonal antibody. Alternative aldehyde-reactive groups are for example, the semicarbazide, the thiosemicarbazide, and the O-substituted hydroxylamine functionalities.

Assembly of conjugate molecules of the invention is not restricted to the sequence outlined in Scheme II. The Hu:CT-M-01 antibody can be first modified to contain a thiol group, which is then reacted with the antitumour antibiotics useful in the invention in accordance with Scheme III below:

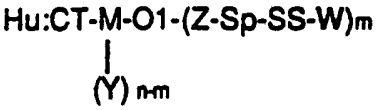
Scheme III



wherein Hu:CT-M-O1, Y, Q, Sp, W, *n*, and *m* are as hereinbefore defined, and P is hydrogen or 2-(pyridylthio), with the proviso that when Y is a thiol derived from a backbone amino acid residue of Hu:CT-M-01, Z-Sp taken together is a covalent bond.

As an example, and with references to Scheme III, above, the Hu:CT-M-01 monoclonal antibody can be reacted with 3-(2-dithiopyridyl)propionic acid hydroxysuccinimide ester to modify the protein through lysine residues (Y=NH_2, n=50-100, Q= -CO_2Su, Sp=-CH_2CH_2-, P=2-pyridylthio). Following reduction with, for example, dithiothreitol, an intermediate is generated (Z=-NHCO-, Sp=-CH_2CH_2-, P=H, m=1-15) which can be reacted with the antitumour antibiotics to generate the subject immunoconjugates. Similarly, 2-iminothiolane can be reacted with Hu:CT-M-01 to introduce thiol groups onto the surface of the protein directly, without requiring a reduction step (Z=-NHCO-, Sp=-$(CH_2)_3$-, P=H, m=1-15), and this intermediate can be reacted with the CH_3-SSS-W as before. Alternatively, sulfhydryl groups inherent within the structure of Hu:CT-M-01 in dimeric form as cystine residues can be used to participate in the reaction of Scheme III directly. Such sulfhydryls are traditionally exposed by a combination of enzymatic digestion and reduction of native monoclonal antibodies (Hu:CT-M-01=Fab' fragment, Z-Sp=Bond, Y=SH).

A preferred embodiment of the present invention is a protein-drug conjugate of the formula:

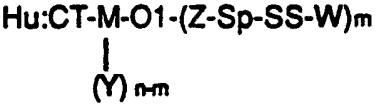


prepared from the class of antitumour antibiotics designated LL-E33288 (CH_3-SSS-W) comprising:

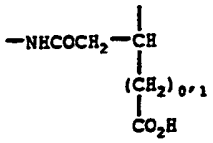
displacing the dithiomethyl moiety with a compound of formula Q-Sp-SH, wherein Sp is straight or branched-chain divalent or trivalent (C_2-C_{10}) radicals or divalent or trivalent (C_2-C_5) arylalkyl or heteroarylalkyl radicals, wherein if Sp is a trivalent radical, it can be additionally substituted by amino, heteroarylamino, hydroxy, or thiol groups; and Q is carboxyl, lower alkyldicarboxyl anhydride, -CO_2Su, -$CONHNH_2$, or

to produce an intermediate of general formula Q-Sp-SS-W, wherein Q, Sp, and W are as hereinbefore defined, and

reacting Q-Sp-SS-W with a molecule of the formula Hu:CT-M-01-$(Y)_n$ wherein Y is a side-chain amino group on the antibody, or an aldehyde generated by oxidation of the carbohydrate groups of the antibody, and *n* is an integer of from 1 to 100, to produce a compound of the formula:



wherein Y, Sp, W, and *n* are as hereinbefore defined, and Z is formed from covalent reaction of the groups Q and Y directly or after subsequent reduction, and Z is -CONH-, -CONHN=CH-, -$CONHNHCH_2$-, or



and *m* is 0.1 to 15.

The present invention also includes therapeutic and diagnostic compositions containing the HAM of the invention, particularly a conjugate molecule comprising a HAM conjugated to an effector or reporter molecule and uses of such compositions in therapy and diagnosis. Such therapeutic and diagnostic compositions typically comprise a HAM according to the invention together with a pharmaceutically acceptable excipient, diluent or carrier, e.g. for *in vivo* use.

Therapeutic and diagnostic uses typically comprise administering a pharmaceutically effective amount of a HAM according to the invention to a human subject. The exact dose to be administered will vary according to the intended use of the HAM and on the age and condition of the patient but may be typically varied from about 0.1mg to 1000mg, for example from about 1mg to 500 mg. The HAM may be administered as a single dose, or in a continuous manner over a period of time. Doses may be repeated as appropriate. The HAM may be formulated in accordance with conventional practice for administration by any suitable route, and may generally be in a liquid form [e.g. a solution of the antibody in a sterile physiologically acceptable buffer] for administration by for example an intravenous, intraperitoneal or intramuscular route.

In the HAM of the first aspect of the invention and the process of the second aspect of the invention, the heavy and light chain variable domains of the HAM may comprise either the entire variable domains of the CTMO1 MAb or may comprise framework regions of a human variable domain having grafted thereon one, two or all three of the CDRs of the CTMO1 MAb. Thus, the HAM may comprise a chimeric humanised antibody or a CDR-grafted humanised antibody.

When the HAM is a CDR-grafted humanised antibody, in addition to the CDRs, specific variable region framework residues may be altered to correspond to non-human, i.e. the CTMO1 mouse, residues. Preferably, the CDR-grafted humanised antibodies of the present invention include CDR-grafted humanised antibodies as defined in our International Patent Specification WO-A-91/09967. The disclosure of WO-A-91/09967 is incorporated herein by reference.

Preferably, the CDRs of the light chain correspond to the Kabat CTMO1 MAb CDRs at CDR1 (residues 24-34) and CDR2 (residues 50-56) and to the structural loop residues (residues 91-96) or Kabat CTMO1 MAb CDR residues (residues 89-97) in CDR3. (The residue designations given above and elsewhere in the present application are numbered according to the Kabat numbering system [16]). In addition, the light chain may have mouse CTMO1 residues at one or more of residues 1, 2, 3, 36, 37, 45, 48, 49, 60, 63, 70, 84, 85, 87 and 108. In preferred embodiments, when the human framework used is EU, the light chain comprises Kabat CTMO1 MAb CDRs at all of CDR1, CDR2 and CDR3 and preferably additional CTM01 residues at positions 3, 36, 37, 45, 48, 63 and 108, or especially additional CTMO1 residues at positions 3, 36, 63 and 108 only.

Preferably, the CDRs of the heavy chain correspond to the Kabat CTMO1 MAb CDRs at all of CDR1 (26 to 35), CDR2 (50 to 65) and CDR3 (94 to 100). In addition, the heavy chain may have mouse CTMO1 residues at one or more of residues, 2, 6, 23, 37, 48, 49, 67, 69, 73, 76, 78, 80, 88, 91 and 94. In particularly preferred embodiments, when the human framework used is EU, the heavy chain framework comprises additional CTM01 MAb residues at positions 2, 37, 71 and 73, and especially in addition at positions 48, 67 and 69.

In addition, EU has a particularly idiosyncratic J region between residues 103 to 113 and it may be useful to include the murine amino acids, a consensus human J region or a suitable combination of both at residues 103 to 108 inclusive. When the EU framework is used,

preferably heavy chain residues 94, 103, 104, 105 and 107 are murine residues, since in the case of these residues, the murine sequence is more frequently found in human VH sequences than the EU residues.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention is now described, by way of example only, with reference to the accompanying drawings, in which:

Figure 1 is a schematic diagram of plasmid pRR62;
Figure 2 is a schematic diagram of plasmid pAL41;
Figure 3 is a schematic diagram of plasmid pMRR017;
Figure 4 is a schematic diagram of plasmid pHMC34;
Figure 5 is a schematic diagram of plasmid pMRR011;
Figure 6 is a schematic diagram of plasmid pHMC32;
Figure 7 is a schematic diagram of plasmid pMRR022;
Figure 8 is a schematic diagram of plasmid pMRR014;
Figure 9 is a schematic diagram of plasmid pHMC33;
Figure 10 is a schematic diagram of plasmid pMRR001;
Figure 11 is a schematic diagram of plasmid pHMC35;
Figure 12 is a schematic diagram of plasmid pHMC38;
Figure 13 is a schematic diagram of plasmid pHMC40;
Figure 14 is a schematic diagram of plasmid pHMC41;
Figure 15 is a schematic diagram of plasmid pHMC42;
Figure 16 shows the alignment of oligonucleotides H1 to H8 in the formation of the gH1 coding sequence;
Figure 17 is a schematic diagram of plasmid pAL51;
Figure 18 is a schematic diagram of plasmid pAL52;
Figure 19 is a schematic diagram of plasmid pMRR010;
Figure 20 is a schematic diagram of plasmid pAL47;
Figure 21 is a schematic diagram of plasmid pAL48;
Figure 22 is a graph of a direct binding ELISA on transiently expressed chimeric antibodies;
Figure 23 is a graph of a direct binding ELISA on transiently expressed CDR-grafted antibodies; and
Figure 24 is a graph of a competition EIA on transiently expressed chimeric and CDR-grafted antibodies.

Figure 25 is a graph comparing the effects on tumour size obtained by treating nude mice implanted with a human ovarian xenograft tumour with a humanised CDR grafted CTMO1 and a murine CTMO1 antibody each conjugated to the hydroxysuccinimide derivative of 4-mercapto-4-methyl-pentanoic acid disulphide of N-acetyl calicheamicin $\gamma 1^{I}$.

DESCRIPTION OF SPECIFIC EMBODIMENTS

The following description of certain embodiments of the invention is provided by way of example only and is not to be regarded as placing any limitation on the scope of the protection claimed.

MOLECULAR CLONING AND CONSTRUCTION OF THE CTMO1 CHIMERIC HEAVY CHAIN

The heavy chain variable domain of CTMO1 was cloned using the polymerase chain reaction. This enabled the construction of the chimeric version in a single step as described below.

Polyadenylated RNA was isolated from the CTMO1 hybridoma cell line using the guanidinium isothiocyanate/lithium chloride method [17]. Double stranded cDNA was synthesised and used as a template for PCR amplification of the VH gene. A set of twenty four 5' forward primers were synthesised to complement a sequence within the murine leader sequence of VH domains [16] and to introduce a BstEII restriction site. A set of twelve 3' reverse primers was synthesised to complement the framework 4 region of VH [20] and included an ApaI restriction site.

The sequence of the basic 5' primer is given in the Sequence Listing as ID No. 3. The set of twenty four primers was based on this primer as follows. In one group of twelve primers, residue 27 remained as C. In three subgroups of four primers, residue 25 either remained as G or is altered to C or T. In each subgroup, the four primers differed at residue 28, which was A, C, G or T. In the subgroups where residue 25 is C or T, the sixth amino acid is His.

In the second group of twelve primers, residue 27 is changed to G. In three subgroups of four primers, residue 25 either remains as G or is altered to C or T. In each subgroup, the four primers differed at residue 28, which was A, C, G or T. Where residue 25 is C or G, amino acid 6 is Gln and where residue 25 is T, amino acid residue G is His. Where residue 28 is T or C, amino acid residue 7 is Cys. Where residue 28 is G, amino acid residue 7 is Trp.

The sequence of the basic 3' PCR primer is given in the Sequence Listing as ID No. 4. The set of twelve primers was based on this primer as follows. Residue 5 could remain as G or could be altered to A or T. Residue 11 either remains as A or is altered to G. Residue 12 either remains as A or is altered to C.

PCR amplification of CTMO1 VH was carried out using the following conditions:

10 pmoles each primer; 20 ng cDNA; 0.5 U Taq polymerase; 94°C 1 min; 50 °C 2 min; 72°C 3 min; for 40 cycles.

The PCR amplified VH fragment was restricted with BstEII and ApaI and ligated to an adaptor to reconstruct the leader sequence and add a 5' HindIII restriction enzyme site. The sequence of the adaptor used is given in the Sequence Listing as Sequence ID No. 5 and codes in part for the leader amino acid sequence of the VH domain of the murine anti TAG-72 monoclonal antibody B72.3 (WO-A-89/01783).

The adapted fragment was then cloned into the HindIII/ApaI sites of the vector pE1004 to give plasmid pRR62 shown in Figure 1. Plasmid pRR62 consists of an SV40 origin of replication followed by the hCMV-MIE promoter/enhancer region. The promoter/enhancer controls a nucleotide sequence encoding a chimeric heavy chain comprising the CTMO1 heavy chain variable domain fused to human g4 constant domains. Downstream of the coding sequence is a poly A site and gpt gene.

The heavy chain variable region of several independent clones of pRR62 were sequenced. The DNA sequence and deduced amino sequence for CTMO1 VH are given in Sequence ID No. 1.

MOLECULAR CLONING AND CONSTRUCTION OF THE CTMO1 CHIMERIC LIGHT CHAIN

Polyadenylated RNA was isolated from the CTMO1 hybridoma cell line using the guanidinium isothiocyanate/lithium chloride method [17]. Double stranded cDNA was synthesised [21] and a cDNA library was constructed in plasmid pSP64 [22] using EcoRI linkers. A screening probe was synthesised, complementary to mouse immunoglobulin light chain constant region by PCR amplification. The light chain probe was a 318 bp PCR fragment encoding the mouse kappa light chain constant region [23].

The probe was radio-labelled ($g^{32}P$) ATP by random hexanucleotide priming and was used to screen the cDNA library.

The clone which encoded the complete leader, variable and constant domains of light chain was isolated and designated as pRB63.

A fragment of pRB63, which encodes the variable domain of the light chain was recovered by PCR amplification. The PCR primers introduced a BstbI and SpII restriction sites at the 5' and 3' ends of the VL region respectively to enable subsequent cloning of the fragment.

The PCR amplified fragment of plasmid pRB63 was restricted with BstbI/SpII and ligated between the BstbI/SpII sites of plasmid pMRR010 to produce plasmid pAL41, which is shown in Figure 2. Plasmid pAL41 consists of a glutamine synthetase cDNA having downstream of it the hCMV-MIE promoter/enhancer region. The promoter/enhancer region controls a nucleotide sequence encoding a chimeric light chain comprising the CTMO1 light chain variable domain fused to a human CK constant domain. Downstream of the coding sequence is a poly A site.

Nucleotide sequence analysis was carried out according to the chain termination procedure [24]. The VH coding sequence insert in pRR62 and the VL coding sequence insert in pAL41 were fully sequenced. The DNA and predicted amino acid sequences for the unprocessed variable domains of the CTM01 heavy and light chains are shown in the Sequence Listing appended to the end of the description as Sequence ID No. 1 and No. 2 respectively.

Sequence No. 1 shows the sequence coding for the VH domain and the predicted amino acid sequence. The leader sequence for the heavy chain runs from residue 1 to residue 19 as shown in Sequence No. 1. Sequence No. 2 shows the sequence coding for the VL domain together with the predicted amino acid sequence. The leader sequence for the light chain runs from residue 1 to residue 20 as shown in Sequence No. 2. Examination of the derived amino acid sequences revealed considerable homology with other characterised immunoglobulin genes. The CTMO1 MAb was confirmed to be an IgG1-kappa antibody.

PREPARATION OF CHIMERIC ANTIBODY PRODUCTS

CHIMERIC LIGHT CHAIN VECTOR

A ClaI-EcoRI fragment of pAL41 carrying the hCMV promoter and chimeric light chain was cloned into plasmid pMRR017 which is shown in Figure 3. Plasmid pMRR017 has a GS mini gene (WO-A-87/04462), hCMV-MIE promoter/enhancer region, a polylinker sequence and a poly A site. This produced plasmid pHMC34, which is shown in Figure 4. In plasmid pHMC34, the chimeric light chain gene is under the control of the hCMV-MIE promoter/enhancer sequence.

CHIMERIC HEAVY CHAIN VECTORS

IgG1 CONSTRUCT

A HindIII-ApaI fragment containing the sequence encoding the VH domain was excised from plasmid pRR62 (Figure 1). This fragment was inserted between the HindIII and ApaI sites of plasmid pMRR011. Plasmid pMRR011 is shown in Figure 5 and comprises an hCMV-MIE

promoter/enhancer region, an SV40 polyadenylation sequence, a gpt gene and a sequence encoding a human IgG1 heavy chain lacking a variable domain. The plasmid thus produced, pHMC32, is shown in Figure 6 and has a chimeric heavy chain coding sequence under the control of the hCMV-MIE promoter/enhancer. The chimeric heavy chain has the VH domain from the CTM01 MAb fused to human IgG1 constant domains.

IgG2 CONSTRUCT

The HindIII-ApaI fragment of pRR62 (Figure 1) was inserted between the HindIII and ApaI sites of a plasmid containing an hCMV-MIE promoter, a polylinker site and a nucleotide coding sequence which encodes the three constant domains of a human IgG2 antibody. This yielded plasmid pMRR022 which encodes a chimeric heavy chain having the CTM01 variable domain linked to the human IgG2 constant domains.

IgG4 CONSTRUCT

The HindIII-ApaI fragment of pRR62 (Figure 1) was inserted between the HindIII and ApaI sites of plasmid pMRR014 to produce plasmid pHMC33. Plasmids pMRR014 and pHMC33 are shown in Figure 8 and 9 respectively. Plasmid pMRR014 has an hCMV-MIE promoter, a polylinker site and a nucleotide coding sequence which encodes the three constant domains of a human IgG4 antibody. Plasmid pHMC33 is identical to plasmid pHMC32 except that the coding sequence encodes a chimeric heavy chain having the CTM01 variable domain and human IgG4 constant domains in place of the human IgG1 constant domains.

ALTERED IgG4 CONSTRUCT

The HindIII-ApaI fragment was reisolated from plasmid pHMC33. Plasmid pMRR001 shown in Figure 10 was digested with HindIII and ApaI. The large fragment was isolated and ligated to the HindIII-ApaI fragment of pHMC33 to produce plasmid pHMC35, shown in Figure 11. Plasmid pHMC35 is almost identical to plasmid pHMC32 except that the coding sequence encodes a chimeric heavy chain having the

CTM01 variable domain and altered human IgG4 (hereinafter referred to as IgG4P) constant domains in place of the human IgG1 constant domains.

The alteration in the constant domains comprises a change of a serine residue in the hinge region at position 241 to a proline residue. This change advantageously abolished the formation of an 80 KD half antibody which otherwise occasionally is formed with IgG4 constant domains.

CHIMERIC HEAVY AND LIGHT CHAIN VECTORS

Vectors were constructed having operons coding for both heavy and light chains within the same vector.

A NotI-SalI fragment carrying the hCMV-MIE promoter/enhancer, the chimeric light chain encoding sequence and the SV40 poly A site together with the GS mini gene was excised from plasmid pHMC34 (Figure 4). A NotI-HindIII fragment carrying the hCMV-MIE promoter/enhancer was excised from plasmid pHMC35 (Figure 11). A HindIII-SalI fragment carrying the altered IgG4 heavy chain coding sequence and SV40 poly A site was excised from plasmid pHMC35 (Figure 11). These three fragments were ligated together to produce plasmid pHMC38, which is shown in Figure 12, and codes for expression of chimeric light chain together with the altered IgG4 chimeric heavy chain.

Plasmids pHMC32, pMRR022 and pHMC33 were digested with HindIII and EcoRI and the fragments containing the chimeric heavy chain encoding sequences were isolated. The isolated fragments were each ligated with the large HindIII-SalI fragment of pHMC38 (Figure 12) and an EcoRI-SalI fragment comprising the SV40 poly A region. The ligations produced plasmids pHMC40, pHMC41 and pHMC42 (shown in Figures 13 to 15 respectively). pHMC40 encodes a heavy chain having IgG1 constant domains. pHMC41 encodes IgG2 constant domains and pHMC42 encodes IgG4 constant domains.

PREPARATION OF CDR-GRAFTED ANTIBODY PRODUCTS

It was decided to use the EU human antibody framework [16] for carrying out the CDR-grafting. The strategy followed for CDR-grafting was as set out in our International Patent Specification No. WO-A-91/09967.

Two CDR-grafted heavy chains were designed. In the first, gH1, all three CDRs [as defined by Kabat, ref. 16] were changed to murine residues. In addition, residues 2, 37, 71, 73, 94, 103, 104, 105 and 107, which are outside the Kabat CDRs, were also changed to murine residues. In the second, gH2, in addition to those murine residues in gH1, residues 48, 67 and 69 were changed to murine residues with a view to improving packing of the VH domain.

Two CDR-grafted light chains were also designed. In the first, gL1, all three CDRs [as defined by Kabat, ref. 16] were changed to murine residues. In addition residues 3, 36, 63 and 108, which are outside the Kabat CDRs, were changed to murine resides. In the second, gL2, in addition to those murine residues in gL1, residues 37, 45 and 48 were changed to murine residues with a view to improving packing.

A nucleotide sequence coding for the gH1 variable domain was produced by oligonucleotide assembly using oligonucleotides H1 to H8. The sequences for these oligonucleotides are given in the Sequence Listing at the end of the description under Sequence ID Nos. 6 to 13. The way in which these oligonucleotides are assembled to produce the gH1 coding sequence is shown in Figure 16. The amino acid sequence coded for by this gH1 sequence is shown in the sequence listing under Sequence ID No. 14.

A nucleotide sequence coding for the gH2 variable domain was also produced by oligonucleotide assembly using oligonucleotides H1, H2, H3A, H4, H5, H6A, H7 and H8. Oligonucleotide H3A differs from oligonucleotide H3 (Sequence ID No. 8) in that residues 55 to 57 have been changed from GTG to GCA and residues 61 to 63 have been changed from ATT to CTG. Oligonucleotide H6A differs from oligonucleotide H6 (Sequence ID No. 11) in that residues 70 to 72

have been changed from TAC to TAA. Thus, the gH2 variable domain encodes the same sequence as is shown under Sequence ID No. 14, except that at residue 67, MET has been changed to ILE; at residue 87, VAL has been changed to ALA; and at residue 89, ILE has been changed to LEU.

A nucleotide sequence coding for the gL1 variable domain was produced by oligonucleotide assembly using oligonucleotides L1 to L8. The sequences for these oligonucleotides are given in the Sequence Listing at the end of the description under Sequence ID Nos. 15 to 22. The way in which these nucleotides are assembled is similar to that shown in Figure 16 for the gH1 coding sequence (except that L is substituted for H). The amino acid sequence coded for by the assembled gL1 variable domain coding sequence is shown in the Sequence Listing under Sequence ID No. 20.

A nucleotide sequence coding for the gL2 variable domain was produced by oligonucleotide assembly using oligonucleotides L1, L2A, L3A and L4 to L8. Oligonucleotide L2A differs from oligonucleotide L2 (Sequence ID No. 16) in that residues 28 to 30 have been changed from CAG to GTA. Oligonucleotide L3A differs from oligonucleotide L3 (Sequence ID No. 17) in that residues 25 - 27 have been changed from CAG to CTC, residues 49 - 52 have been changed from AAG to CAG and residues 59 - 61 have been changed from CAT to ATC. Thus, the gL2 variable domain encodes the same sequence as is shown under Sequence ID NO. 23, except that: at residue 23, Gln has been changed to Val; at residue 62, G1n has been changed to Leu; at residue 60, Lys has been changed to G1n; and at residue 73, Met has been changed to Ile.

For gene assembly 1 pmol of H2 - H7 or L2 - L7 was mixed with 10 pmol or H1 and H8 or L1 and L8 in a 100 ml reaction with 5U Taq polymerase. A PCR reaction was done using 30 cycles (95°C, 1 min. 50°C 1 min; 72°C 1 min). The resulting fragments were cut with HindIII and ApaI for VL with Bstb1 and SPII for VH.

The nucleotide sequences coding for gH1 and gH2 were cloned as HindIII-ApaI fragments into plasmid pMRR014 (Figure 8) to produce plasmids pAL51 and pAL52 (Figure 17 and 18 respectively).

The nucleotide sequences coding for gL1 and gL2 were cloned as HindIII-ApaI fragments into plasmid pMRR010 (Figure 19) to produce plasmids pAL47 and pAL48 (Figures 20 and 21 respectively).

TRANSIENT EXPRESSION OF CHIMERIC/CHIMERIC OR CDR-GRAFTED/CHIMERIC ANTIBODIES

The following plasmids:

pHMC38, pHMC40, pHMC41 and pHMC42

and the following pairs of plasmids:

pAL47, pHMC33; pAL48, pHMC33; pAL51, pAL41; pAL52, pAL41; and pAL48, pAL41;

were each transfected or cotransfected into CHO-L761h cells for transient expression.

Assembly ELISA assays on culture supernatants resulting from the single transfected cells showed that they contained assembled antibody.

The assembly ELISA assay for quantifying antibody yields used microwell plates coated with a goat $F(ab')_2$ anti-human IgGFc. Following incubation with transfected culture supernatants, bound chimeric or CDR-grafted antibody was revealed with a horseradish peroxidase (HRP)-conjugated murine anti-human IgK antibody using tetramethyl benzidine (TMB) as the substrate. Concentrations of chimeric or CDR-grafted whole antibody in the samples were interpolated from a calibration curve generated from serial dilutions of purified chimeric B72.3 $\gamma 4$ antibody [25].

BINDING ACTIVITY OF TRANSIENTLY EXPRESSED CHIMERIC OR CDR-GRAFTED ANTIBODIES

Direct binding ELISA assays for determining the binding activity of the transiently expressed antibodies were carried out as follows.

An affinity column was prepared by attaching the CTM01 MAb to a suitable chromatographic medium in conventional manner. In a first method, pooled human urine samples were applied directly to the affinity column. In a second method, human milk was subjected to low speed centrifugation to separate the cream from skimmed milk. The skimmed milk was then subjected to high speed centrifugation to produce an aqueous and a lipid component. The aqueous component was applied to the affinity column.

Once the affinity column was loaded, by either of the two methods, column fractions were eluted at high and low pHs, neutralised and assayed for reactivity with the CTM01 MAb. Fractions showing reactivity were pooled and dialysed. The pooled fractions contained the polymorphic epithelial mucin (PEM) recognised by the CTM01 MAb.

Microwell plates were coated with PEM obtained as described above. The microwells were then incubated with serial dilutions of culture supernatants. Binding of chimeric or CDR-grafted antibody was revealed and quantified by use of an HRP-conjugated murine anti-human IgK antibody.

The results of direct binding ELISA assays on the supernatants from singly transfected cells are shown in Figure 22. These assays confirm that all the supernatants contained antibodies capable of binding to PEM. No significant differences in binding activity were observed.

The direct binding ELISA assays on the supernatants from doubly transfected cells confirmed that the supernatants contained antibodies capable of binding to PEM and that the chimeric/chimeric antibody bound better than any of the CDR-grafted/chimeric antibodies.

A competition binding assay was carried out using polystyrene beads coated with PEM obtained as described above. CTM01 MAb was radiolabelled with 125I and was used to compete with the antibody produced by the pHMC40 (IgG1) transfected cells. The potency of the chimeric antibody was 84-102% that of the CTM01 MAb.

TRANSIENT EXPRESSION OF CDR-GRAFTED/CDR-GRAFTED ANTIBODIES

The following pairs of plasmids:

pAL47, pAL51; pAL47, pAL52; pAL48, pAL51; and pAL48, pAL52;

were cotransfected into CHO-L761 cells.

Direct binding assays were carried out on the culture supernatants produced by the doubly transfected cell lines.

The results of these assays are shown in Figure 23, together with some results for chimeric/CDR-grafted antibodies.

From all the direct binding assays referred to above, it can be determined that the order of binding activity of the various antibodies produced by transient expression is as follows:

cLcH3>gL1ch = gL1gH2>cLgH2 = gL2H2 = gL1gH1 = gL2cH>gL2gH1.

(wherein:cL = chimeric light chain;
cH = chimeric heavy chain
gL1 = CDR-grafted light chain with lowest number of amino acid changes;
gL2 = CDR-grafted light chain with highest number of amino acid changes;
gH1 = CDR-grafted heavy chain with lowest number of amino acid changes;
gH2 = CDR-grafted heavy chain with highest number of amino acid changes).

The more active variants (cLcH, gL1cH, gL1gH2 and gL2gH2) together with the CTM01 MAb were tested in a competition enzyme immunoassay (EIA). Microwell plates were coated with PEM obtained as described above. The CTM01 MAb was biotinylated and was used to compete with the four variants referred to above. Bound biotinylated

CTM01 MAb was revealed and quantified using a streptavidin-HRP conjugate and TMB.

The results of the competition EIA are shown in Figure 24, which shows the same ranking of binding activity as set out above, except that the gL1cH combination shows greater activity than the cLcH combination.

It can thus be seen that chimeric, chimeric/CDR-grafted and CDR-grafted antibodies which recognise the same antigen as the CTM01 MAb have successfully been produced.

IN VITRO CELL BINDING AND INTERNALISATION OF CDR-GRAFTED CTMO1 ANTIBODIES

Stable NSO cell lines expressing gL1gH2lgG2 CTMO1 (hereinafter hu1:CTMO1) and gL1gH2lgG4P CTMO1 (hereinafter hu:CTMO1) antibody variants were made by transfecting into NSO cells by electroporation double gene expression plasmids assembled by ligating the large (7.8 kbp) Not1/BamH1 fragment of pAL47 to the 2.4 kbp Not1/Apa1 fragment from pAL52 and either a 1.9Kbp BamH1/Apa1 (partial) fragment carrying the lgG2 constant domains or a 2kbp Apa1/BamH1 fragment carrying the lgG4P constant domains as appropriate.

Antibody, purified from the supernatant of each cultured cell line by protein-A sepharose chromatography was radiolabelled (^{125}I) and incubated using a conventional continuous exposure method with either MX-1 or MCF-7 breast carcinoma cells. Radiolabelled murine CTM01 was used in all tests as a comparison. All antibodies were incubated at 2µg/million cells. The total binding of antibodies to the cells and the peak net uptake of the antibodies by the cells was determined. The results are shown in Table 1 below. With both cell lines each CDR grafted antibody exhibited better binding and internatlisation than the murine form.

TABLE 1

Antibody	Cell Line	Total Binding, 0° (molecules/cell)	Peak Net Uptake (molecules/cell)
hu1:CTMO1	MCF-7	650,000	150,000
hu:CTMO1	MCF-7	450,000	90,000
Murine CTMO1	MCF7	300,000	70,000
hu1:CTMO1	MX-1	1,200,000	150,000
hu:CTMO1	MX-1	1,100,000	150,000
Murine CTMO1	MX-1	800,000	80,000

IN VIVO ANTI-TUMOUR ACTIVITY OF A CONJUGATE OF hu:CTMO1 AND AN ANTI-TUMOUR ANTIBIOTIC

hu:CTMO1 was conjugated to the hydroxysuccinimide derivative of 4-mercapto-4-methyl-pentanoic acid disulphide of N-acetyl calicheamicin γ_1^I as follows:

SYNTHESIS OF THE 4-MERCAPTO-4-METHYL-PENTANOIC ACID DISULPHIDE DERIVATIVE OF N-ACETYL CALICHEAMICIN γ_1^I

To N-acetyl calicheamicin γ_1^I [US Patent No. 5079233] at a concentration of 2 mg/mL in acetonitrile at -15°C was added 5 molar equivalents of 4-mercapto-4-methyl-pentanoic acid and 6 molar equivalents of triethylamine. After 24 hours at -15°C the reaction was checked by C_{18}-HPLC. [If the reaction is incomplete, additional amounts of 4-mercapto-4-methyl-pentanoic acid and triethylamine are added]. Upon completion of the reaction the volatile organics were evaporated under reduced pressure and the crude product was chromatographed on Bio-Sil A using a gradient of 1 to 5% methanol in chloroform. Pure fractions as assessed by tlc were pooled and evaporated to a glass. The ^{1}H-NMR of the product was similar to N-acetyl calicheamicin γ_1^I, but was missing the absorbance for -SSSMe and exhibits absorbances for the methylpentanoic acid moiety as expected. FAB-MS gave m/z = 1478 (M + H) and 1500 (M + Na).

SYNTHESIS OF THE HYDROXYSUCCINIMIDE DERIVATIVE OF 4-MERCAPTO-4-METHYL-PENTANOIC ACID DISULPHIDE OF N-ACETYL CALICHEAMICIN $\gamma 1^I$

To the 4-mercapto-4-methyl-pentanoic acid disulphide derivative of N-acetyl calicheamicin $\gamma 1^I$ described above at a concentration of 5 mg/mL in acetonitrile at ambient temperature was aded 3 molar equivalents of N-hydroxysuccicinimide and 5 molar equivalents of 1-(3-dimethylamino-propyl)-3-ethylcarbodiimide hydrochloride. After 1 hour the reaction was checked by C_{18}-HPLC. [If the reaction is incomplete, then additional 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride is added]. Upon completion of the reaction the volatile organics were evaporated under reduced pressure and the crude product was chromatographed on Bio-Sil A using a gradient of 0 to 5% methanol in chloroform. Pure fractions as assessed by tlc were pooled and stripped to a glass. The ^{1}H-NMR was similar to that of the product described above, but with absorbances present for succinimide, as expected. FAB-MS gave m/z = 1575 (M + H) and 1597 (M + Na).

SYNTHESIS OF hu:CTM01 CONJUGATE USING THE HYDROXYSUCCINIMIDE DERIVATIVE OF 4-MERCAPTO-4-METHYL-PENTANOIC ACID DISULPHIDE OF N-ACETYL CALICHEAMICIN $\gamma 1^I$

To hu:CTM01 in phosphate buffer at a pH of about 7.4 was added 2-6 molar equivalents of the hydroxysuccinimide derivative of 4-mercapto-4-methyl-pentanoic acid disulphide of n-acetyl calicheamicin $\gamma 1^I$, described above, in dimethylformamide (DMF) such that the final concentration of DMF was 10-15%. After completion of the reaction (2-24 hours) the low-molecular-weight organic material was removed by passing through a desalting column using pH 7.4 phosphate buffer. The product was further purified by chromatography on a gel exclusion column and concentrated to give a monomeric product with an average loading of 1-3 molecules of calicheamicin derivative per molecule of antibody.

IN VIVO TEST FOR ANTITUMOUR ACTIVITY

The human ovarian xenograft tumour, OvCar3, implanted subcutaneously in nude mice was used as a test system to study the efficacy of the hu:CTM01 conjugate in vivo. A murine CTMO1 conjugate containing the same calicheamicin was also tested for comparison. Tumours were implanted subcutaneously into athymic mice and test samples were inoculated intraperitoneally (IP) at several dose levels on a q4 day x 3 schedule, starting 2-3 days after tumour implantation with 6 mice per group and 10 in each control group. Tumour mass was determined by measuring the tumour diameter once weekly during 42 days post tumour implantation. Significant antitumour activity was defined as a sustained 58% inhibition of mean tumour mass compared with untreated controls in groups with grater than 65% survivors. At both the 1 and 3 μg doses of drug equivalents the hu:CTMO1 conjugate showed significant inhibition of tumour growth (Figure 25). No deaths were noted in the 42 days observation period in any test group. In all test groups, n=6, in the control group n=10, error bars = ± Standard Error Mean for each data point.

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SEQUENCE LISTING

SEQUENCE ID NO: 1.
SEQUENCE TYPE: Nucleotide with deduced protein sequence.
SEQUENCE LENGTH: 416 bases.
STRANDEDNESS: Single.
TOPOLOGY: Linear.
MOLECULE TYPE: cDNA.
ORIGINAL SOURCE ORGANISM: Murine.
IMMEDIATE EXPERIMENTAL SOURCE
NAME OF CELL LINE: Hybridoma CTM01.
PROPERTIES: Coding sequence for variable domain of heavy chain of the CTM01 monoclo antibody.
FEATURES: Leader sequence from residues 1 to 19.

ATG GAA TGG AGC TGG GTC TTT CTC TTC TTC CTG TCG GTA ACC ACA GGT
Met Glu Trp Ser Trp Val Phe Leu Phe Phe Leu Ser Val Thr Thr Gly

GTC CAT TGC CAG ATC CAG CTG CAG CAG TCT GGA CCT GAG CTG GTG AAG
Val His Cys Gln Ile Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys

CCT GGG GCT TCA GTG AAG ATA TCC TGC AAG GCT TCT GGC TAC ACC TTC
Pro Gly Ala Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe

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ACT GAC TAC TAT ATA AAC TGG ATG AAG CAG AAG CCT GGA CAG GGA CTT   192
Thr Asp Tyr Tyr Ile Asn Trp Met Lys Gln Lys Pro Gly Gln Gly Leu    64

GAG TGG ATT GGA TGG ATT GAT CCT GGA AGC GGT AAT ACT AAG TAC AAT   240
Glu Trp Ile Gly Trp Ile Asp Pro Gly Ser Gly Asn Thr Lys Tyr Asn    80

GAG AAG TTC AAG GGC AAG GCC ACA TTG ACT GTA GAC ACA TCC TCC AGC   288
Glu Lys Phe Lys Gly Lys Ala Thr Leu Thr Val Asp Thr Ser Ser Ser    96

ACA GCC TAC ATG CAG CTC AGC AGC CTG ACA TCT GAG GAC ACT GCT GTC   336
Thr Ala Tyr Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Thr Ala Val   112

TAT TTC TGT GCA AGA GAG AAA ACG ACC TAT TAC TAT GCT ATG GAC TAC   384
Tyr Phe Cys Ala Arg Glu Lys Ile Thr Tyr Tyr Tyr Ala Met Asp Tyr   128

TGG GGT CAA GGA ACC TCA GTC ACT GTC TCC GC                        416
Trp Gly Gln Gly Thr Ser Val Thr Val Ser Ala                       139
```

SEQUENCE ID NO: 2.
SEQUENCE TYPE: Nucleotide with deduced protein sequence.
SEQUENCE LENGTH: 399 bases.
STRANDEDNESS: Single.
TOPOLOGY: Linear.
MOLECULE TYPE: cDNA.
ORGINAL SOURCE ORGANISM: Murine.
IMMEDIATE EXPERIMENTAL SOURCE
NAME OF CELLINE: Hybridoma CTM01.
PROPERTIES: Coding sequence for variable domain of light chain of the CTM01 monoclonal antibody
FEATURES: Leader sequence from residues 1 to 20.

ATG AGG TGC CTA GCT GAG TTC CTG GGG CTG CTT GTG CTC TGG ATC CCT	48
Met Arg Cys Leu Ala Gly Phe Leu Gly Leu Leu Val Leu Trp Ile Pro	16
GGA GCC ATT GGG GAT ATT GTG ATG ACT CAG GCT GCA CCC TCT GTT CCT	96
Gly Ala Ile Gly Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Pro	32
GTC ACT CCT GGA GAG TCA TTA TCC ATT TCC TGC AGG TCT AGT AAG AGT	144
Val Thr Pro Gly Gly Ser Leu Ser Ile Ser Cys Arg Ser Ser Lys Ser	48
CTC CTT CAT AGT AAT GGC GAC ACT TTC TTG TAT TGG TTC CTG CAG AGG	192
Leu Leu His Ser Asn Gly Asp Thr Phe Leu Tyr Trp Phe Leu Gln Arg	64

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CCA GGC CAG TCT CCT CAA CTC CTG ATA TAT CGG ATG TCC AAC CTT GCC   240
Pro Gly Gln Ser Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala    80

TCC GGA GTC CCA GAC AGG TTC AGT GGC AGT GGG TCA GGA ACT GCT TTC   288
Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe    96

ACA CTG AGA GTC AGT AGA GTG GAG GCT GAG GAT GTG GGT GTT TAT TAC   336
Thr Leu Arg Val Ser Arg Val Glu Ala Gly Asp Phe Gly Val Tyr Tyr   112

TGT ATG CAA CAT CTA GAA TAT CCT TTC ACG TTC GGT GCT GGG ACC AAG   384
Cys Met Gln His Leu Gly Tyr Pro Phe Tyr Phe Gly Ala Gly Thr Lys   128

CTG GAG CTG AAA CGG                                              399
Leu Gly Leu Lys Arg                                              133
```

SEQUENCE ID NO. 3
SEQUENCE TYPE: Nucleotide sequence with corresponding amino acid sequence
SEQUENCE LENGTH: 28 bases
STRANDEDNESS: Single
TOPOLOGY: Linear
MOLECULAR TYPE: Synthesised DNA
PROPERTIES: 5' forward primers for PCR amplification of murine VH domains
FEATURES: Complementary to leader sequence of murine VH domains with introduced BstEII restriction site at residues 7 to 13

GGTGGCG GTA ACC ACA GGT GTC CAG TCA 28
Val Thr Thr Gly Val Gln Ser 7

SEQUENCE ID NO. 4
SEQUENCE TYPE: Nucleotide sequence
SEQUENCE LENGTH: 36 bases
STRANDEDNESS: Single
TOPOLOGY: Linear
MOLECULE TYPE: Synthesised DNA
PROPERTIES: 3' reverse primer for PCR amplification of murine VH domains
FEATURES: Complementary to framework 4 region of murine VH including an ApaI restriction site at residues 25 to 30

AGTGGCAGAG AAGTCGGAGT TGCTTCCCGG GTAGAC 36

SEQUENCE ID NO. 5

SEQUENCE TYPE: Nucleotide sequences with corresponding amino acid sequence

SEQUENCE LENGTH: 50 bases (sense strand) and 51 bases (anti-sense strand)

STRANDEDNESS: Double

TOPOLOGY: Linear

MOLECULE TYPE: Syntheised DNA

PROPERTIES: Leader sequence adaptor for murine VH domain

FEATURES: Comprises a HindIII-BstEII fragment in part coding for the leader sequence of the VH domain of murine monoclonal antibody B72.3

```
AGCTTGCCGC CACC ATG GAA TGG AGC TGG GTC TTT CTC TTC TTC CTG TCG        50
    ACGGCG GTGG TAC CTT ACC TCG ACC CAG AAA GAG AAG AAG GAC AGC CATTG  51
                Met Glu Trp Ser Trp Val Phe Leu Phe Phe Leu Ser        12
```

SEQUENCE ID NO: 6
SEQUENCE TYPE: Nucleotide.
SEQUENCE LENGTH: 21.
STRANDEDNESS: Single.
TOPOLOGY: Linear.
MOLECULE TYPE: Synthetic oligonucleotide
PROPERTIES: Used for assembly of CDR-grafted heavy chain
FEATURES: HindIII site at residues 7-12

GCGCGCAAGC TTGCCGCCAC C 21

SEQUENCE ID NO: 7.
SEQUENCE TYPE: Nucleotide
SEQUENCE LENGTH: 96.
STRANDEDNESS: Single.
TOPOLOGY: Linear.
MOLECULE TYPE: Synthetic oligonucleotide.
PROPERTIES: Used for assembly of CDR-grafted heavy chain.

TCTCAGATTC AGCTGGTGCA GTCTGGAGCA GAGGTGAAGA AGCCTGGATC 50
TTCTGTGAAG GTGTCTTGTA AGGCATCTGG ATACACCTTC ACCGAC 96

SEQUENCE ID NO: 8.
SEQUENCE TYPE: Nucleotide.
SEQUENCE LENGTH: 96.
STRANDEDNESS: Single.
TOPOLOGY: Linear.
MOLECULE TYPE: Synthetic oligonucleotide.
PROPERTIES: Used for assembly of CDR-grafted heavy chain.

TGGATTGACC CTGGATCTGG AAATACAAAG TACAATGAGA AGTTCAAGGG 50
AAGAGTGACA ATTACAGTGG ACACATCCAC GAATACCGCC TACATG 96

SEQUENCE ID NO: 9.
SEQUENCE TYPE: Nucleotide.
SEQUENCE LENGTH: 89.
STRANDEDNESS: Single.
TOPOLOGY: Linear.
MOLECULE TYPE: Synthetic oligonucleotide.
PROPERTIES: Used for assembly of CDR-grafted heavy chain.
FEATURES: ApaI site at residues 78-83

GAGAAGACCA CCTACTACTA CGCAATGGAC TACTGGGGAC AGGGAACACT 50
GGTGACAGTG TCTTCTGCCT CAACGAAGGG CCCGCGCGC 89

SEQUENCE ID NO: 10.
SEQUENCE TYPE: Nucleotide.
SEQUENCE LENGTH: 96.
STRANDEDNESS: Single.
TOPOLOGY: Linear.
MOLECULE TYPE: Synthetic oligonucleotide.
PROPERTIES: Used for assembly of CDR-grafted heavy chain.

CTGCACCAGC TGAATCTGAG AATGGACTCC TGTAGTTACT GACAGGAAGA 50
AGAGAAAGAC CCAGCTCCAT TCCATGGTGG CGGCAAGCTT GCGCGC 96

SEQUENCE ID NO: 11.
SEQUENCE TYPE: Nucleotide.
SEQUENCE LENGTH: 96.
STRANDEDNESS: Single.
TOPOLOGY: Linear.
MOLECULE TYPE: Synthetic oligonucleotide.
PROPERTIES: Used for assembly of CDR-grafted heavy chain.

TCCAGATCCA GGGTCAATCC ATCCCATCCA CTCGAGTCCC TGTCCAGGTG 50
CCTGTCTCAT CCAATTAATG TAGTAGTCGG TGAAGGTGTA TCCAGA 96

SEQUENCE ID NO: 12.
SEQUENCE TYPE: Nucleotide.
SEQUENCE LENGTH: 93.
STRANDEDNESS: Single.
TOPOLOGY: Linear.
MOLECULE TYPE: Synthetic oligonucleotide.
PROPERTIES: Used for assembly of CDR-grafted heavy chain.

GTAGTAGTAG GTGGTCTTCT CTCTTGCACA GAAGTAGAAT GCTGTGTCCT 50
CAGATCTCAG AGAAGACAGC TCCATGTAGG CGGTATTCGT GGA 93

SEQUENCE ID NO: 13.
SEQUENCE TYPE: Nucleotide.
SEQUENCE LENGTH: 21.
STRANDEDNESS: Single.
TOPOLOGY: Linear.
MOLECULE TYPE: Synthetic oligonucleotide.
PROPERTIES: Used for assembly of CDR-grafted heavy chain.
FEATURES: ApaI site at residues 7 - 12.

GCGCGCGGGC CCTTCGTTGA G 21

SEQUENCE ID NO: 14.
SEQUENCE TYPE: Amino acid.
SEQUENCE LENGTH: 139.
MOLECULE TYPE: Immunoglobulin heavy chain variable domain.
FEATURES: CDRs at residues 45 - 54, 69 - 85 and 118 - 128

Met Glu Trp Ser Trp Val Phe Leu Phe Phe Leu Ser Val Thr Thr Gly	16
Val His Ser Gln Ile Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys	32
Pro Gly Ser Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe	48
Thr Asp Tyr Tyr Ile Asn Trp Met Arg Gln Ala Pro Gly Gln Gly Leu	64
Glu Trp Met Gly Trp Ile Asp Pro Gly Ser Gly Asn Thr Lys Tyr Asn	80
Glu Lys Phe Lys Gly Arg Val Thr Ile Thr Val Asp Thr Ser Thr Asn	96
Thr Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Phe	112
Tyr Phe Cys Ala Arg Glu Lys Thr Thr Tyr Tyr Tyr Ala Met Asp Tyr	128
Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser	139

SEQUENCE ID NO: 15.
SEQUENCE TYPE: Nucleotide.
SEQUENCE LENGTH: 21.
STRANDEDNESS: Single.
TOPOLOGY: Linear.
MOLECULE TYPE: Synthetic oligonucleotide.
PROPERTIES: Used for assembly of CDR-grafted light chain.
FEATURES: BstBI site at residues 7 to 11

GGACTGTTCG AAGCCGCCAC C 21

SEQUENCE ID NO: 16.
SEQUENCE TYPE: Nucleotide.
SEQUENCE LENGTH: 81.
STRANDEDNESS: Single.
TOPOLOGY: Linear.
MOLECULE TYPE: Synthetic oligonucleotide.
PROPERTIES: Used for assembly of CDR-grafted light chain.

TGGCTTACAG ATGCCAGATG CGATATCCAG ATGACTCAGA GTCCAAGTAC 50
TCTCAGTGCC AGTGTAGGTG ATAGGGTCAC C 81

SEQUENCE ID NO: 17.
SEQUENCE TYPE: Nucleotide.
SEQUENCE LENGTH: 90.
STRANDEDNESS: Single.
TOPOLOGY: Linear.
MOLECULE TYPE: Synthetic oligonucleotide.
PROPERTIES: Used for assembly of CDR-grafted light chain.

GGTGACACCT TCCTCTATTG GTTCCAGCAG AAACCAGGTA AAGCCCCAAA 50
GCTCCTCATG TATAGGATGA GTAACCTCGC CAGTGGTGTA 90

SEQUENCE ID NO: 18.
SEQUENCE TYPE: Nucleotide.
SEQUENCE LENGTH: 99.
STRANDEDNESS: Single.
TOPOLOGY: Linear.
MOLECULE TYPE: Synthetic oligonucleotide.
PROPERTIES: Used for assembly of CDR-grafted light chain

CAGCCAGATG ATTTCGCCAC TTATTATTGT ATGCAGCATC TCGAATATCC 50
ATTCACTTTC GGTCAGGGTA CTAAAGTAGA AGTAAAACGT ACGGGCCGG 99

SEQUENCE ID NO: 19.
SEQUENCE TYPE: Nucleotide.
SEQUENCE LENGTH: 81.
STRANDEDNESS: Single.
TOPOLOGY: Linear.
MOLECULE TYPE: Synthetic oligonucleotide.
PROPERTIES: Used for assembly of CDR-grafted light chain.
FEATURES: BstBI site at residues 70 to 75.

GCATCTGGCA TCTGTAAGCC ACAGCAGCAG GAGTCCGAGG ACTTGGGTGG 50
GGACAGACAT GGTGGCGGCT TCGAACAGTC C 81

SEQUENCE ID NO: 20.
SEQUENCE TYPE: Nucleotide.
SEQUENCE LENGTH: 81.
STRANDEDNESS: Single.
TOPOLOGY: Linear.
MOLECULE TYPE: Synthetic oligonucleotide.
PROPERTIES: Used for assembly of CDR-grafted light chain.
CCAATAGAGG AAGGTGTCAC CGTTACTATG GAGGAGACTT TTACTACTCC 50
TACAAGTGAT GGTGACCCTA TCACCTACAC T 81

SEQUENCE ID NO: 21.
SEQUENCE TYPE: Nucleotide.
SEQUENCE LENGTH: 102.
STRANDEDNESS: Single.
TOPOLOGY: Linear.
MOLECULE TYPE: Synthetic oligonucleotide.
PROPERTIES: Used for assembly of CDR-grafted light chain.

AGTGGCGAAA TCATCTGGCT GGAGACTACT GATAGTGAGA GTGAACTCAG 50
TACCACTACC ACTACCACTG AATCTAGATG GTACACCACT GGCGAGGTTA 100
CT 102

SEQUENCE ID NO: 22.
SEQUENCE TYPE: Nucleotide.
SEQUENCE LENGTH: 21.
STRANDEDNESS: Single.
TOPOLOGY: Linear.
MOLECULE TYPE: Synthetic oligonucleotide.
PROPERTIES: Used for assembly of CDR-grafted light chain.
FEATURES: SplI site at residues 7 - 12.
CCGGCCCGTA CGTTTTACTT C 21

SEQUENCE ID NO: 23.
SEQUENCE TYPE: Aminoacid.
SEQUENCE LENGTH: 133.
MOLECULE TYPE: Immunoglobulin light chain variable domain.
FEATURES: CDRs at residues 44 - 59, 75 - 81 and 114 - 122

Met Ser Val Pro Thr Gln Val Leu Gly Leu Leu Leu Leu Trp Leu Thr	16
Asp Ala Arg Cys Asp Ile Gln Met Thr Gln Ser Pro Ser Thr Leu Ser	32
Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Arg Ser Ser Lys Ser	48
Leu Leu His Ser Asn Gly Asp Thr Phe Leu Tyr Trp Phe Gln Gln Lys	64
Pro Gly Lys Ala Pro Lys Leu Leu Met Tyr Arg Met Ser Asn Leu Ala	80
Ser Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Glu Phe	96
Thr Leu Thr Ile Ser Ser Leu Gln Pro Asp Asp Phe Ala Thr Tyr Tyr	112
Cys Met Gln His Leu Glu Tyr Pro Phe Thr Phe Gly Gln Gly Thr Lys	128
Val Glu Val Lys Arg	133

CLAIMS

1. A humanised antibody molecule (HAM) having specificity for human milk fat globule (HMFG) and having an antigen binding site wherein at least one of the complementarity determining regions (CDRs) of the variable domain is derived from the mouse monoclonal antibody CTMO1 (CTMO1 MAb) and the remaining immunoglobulin-derived parts of the HAM are derived from a human immunoglobulin or an analogue thereof.

2. A HAM according to Claim 1 which is a chimeric humanised antibody.

3. A HAM according to Claim 1 which is CDR-grafted humanised antibody.

4. A HAM according to any one of Claims 1-3 when produced by recombinant DNA technology.

5. A HAM according to any one of Claims 1-4, which comprises a complete antibody molecule, or a Fab, Fab', (Fab')2 or Fv fragment, or a single chain antibody fragment.

6. A CDR-grafted HAM according to any one of Claims 3-5 comprising LAY, POM, TUR, TEI, KOL, NEWM, REI or EU human variable region framework sequences for the heavy and/or light chains.

7. A CDR-grafted HAM according to any one of Claims 3-5 comprising EU human variable region framework sequences for both the heavy and light chains.

8. A CDR-grafted HAM according to any one of Claims 3-7 having CTMO1 MAb CDRs at positions 24 to 34 (CDR1), 50 to 56 (CDR2) and 91 to 96 or 89 to 97 (CDR3) of the light chain variable region.

9. A CDR-grafted HAM according to any one of Claims 3-8 additionally comprising CTMO1 MAb residues at one or more of positions 1, 2, 3, 36, 37, 45, 48, 49, 60, 63, 70, 84, 85, 87 and 108 of the light chain variable region.

10. A CDR-grafted HAM according to any one of Claims 3-9 having CTMO1 MAb CDRs at positions 26 to 35 (CDR1), 50 to 65 (CDR2) and 94 to 100 (CDR3) of the heavy chain variable region.

11. A CDR-grafted HAM according to any one of Claims 3-10 additionally having CTM01 MAb residues at any one or more of positions 2, 6, 23, 37, 48, 49, 67, 69, 73, 76, 78, 80, 88, 91 and 94 of the heavy chain variable region.

12. A CDR-grafted HAM according to Claim 7, having CTMO1 MAb CDRs at positions 24 to 34 (CDR1). 50 to 56 (CDR2) and 91 to 96 or 89 to 97 (CDR3) of the light chain variable region, and CTMO1 MAb CDRs at positions 26 to 35 (CDR1), 50 to 65 (CDR2) and 94 to 100 (CDR3) of the heavy chain variable region, and additionally comprising CTMO1 MAb residues at positions 3, 36, 63 and 108, or at positions 3, 36, 37, 45, 48, 63 and 108 of the light chain, and CTMO1 MAb residues at positions 2, 37, 71, 73, 94, 103, 104, 105 and 107, or at positions 2, 37, 48, 67, 69, 71, 73, 94, 103, 104, 105 and 107 of the heavy chain.

13. A CDR-grafted HAM according to Claim 12 wherein the additional CTMO1 MAb residues are at positions 3, 36, 37, 45, 48, 83 and 108 of the light chain, and at positions 2, 37, 48, 67, 69, 71, 73, 94, 103, 104, 105 and 107 of the heavy chain.

14. A CDR-grafted HAM according to Claims 12 or 13 having human IgG heavy chain constant regions or analogues thereof fused to the heavy chain variable domain.

15. A CDR-grafted HAM according to Claim 14 wherein the IgG heavy chain constant regions are human IgG4 constant regions or analogues thereof.

16. A CDR-grafted HAM according to Claim 15 wherein the IgG4 heavy chain constant regions are human IgG4 heavy chain constant regions containing a proline residue at position 241.

17. A CDR-grafted HAM according to Claims 15 or 16 having a human Kappa chain constant region fused to the light chain variable domain.

18. A process for producing a HAM according to any one of Claims 1 to 17 which process comprises:-

(a) producing in an expression vector an operon having a DNA sequence which encodes an antibody heavy or light chain comprising a variable domain wherein at least one of the CDRs of the variable domain is derived from the CTMO1 MAb and the remaining immunoglobulin-derived parts of the antibody chain are derived from a human immunoglobulin;
(b) producing in an expression vector an operon having a DNA sequence which encodes a complementary antibody light or heavy chain comprising a variable domain wherein at least one of the CDRs of the variable domain is derived from the CTMO1 MAb and the remaining immunoglobulin-derived parts of the antibody chain are derived from a human immunoglobulin;
(c) transfecting a host cell with the or each vector; and
(d) culturing the transfected cell line to produce the HAM.

19. A process according to Claim 18, wherein the heavy and light chain encoding sequences are present on the same vector.

20. A process according to Claim 19, wherein the heavy and light chain encoding sequences are present on separate vectors.

21. A process according to any one Claims 18 - 20 for the production of an antibody fragment in which the host cell is a bacterial host cell.

22. A process according to any one of Claims 18 - 20 in which the host cell is a mammalian host cell.

23. A conjugate molecule comprising a HAM according to any one of Claims 1 to 17 conjugated to an effector or reporter molecule.

24. A conjugate moelcule according to Claim 23 wherein the effector molecule is a methyltrithio anti-tumour agent.

25. A conjugate molecule according to Claim 24 wherein the methyltrithio anti-tumour agent is a disulphide analogue of the α_1, $\alpha 2$, α_3, α_4, β_1, β_2, γ_1, δ_1 and pseudoaglycone components of the LL-E33288 complex and derivatives thereof, BBM-1675, FR-900405, FR-900406, PD 114759, PD 115028, CL-1577A, CL-1577B, CL-1577D, CL-1577E and CL 1724 antitumour antibiotics and derivatives thereof.

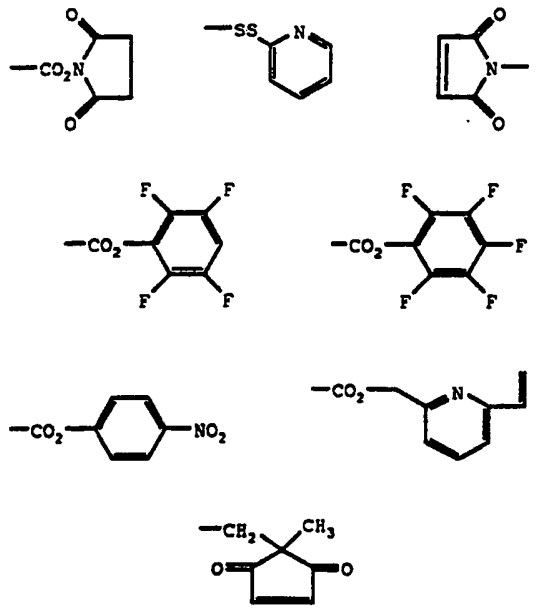
26. A conjugate molecule according to Claim 25 of the formula

$$\begin{array}{c} \text{Hu:CT-M-O1-(Z-Sp-SS-W)}_m \\ \Big| \\ (Y)_{n-m} \end{array}$$

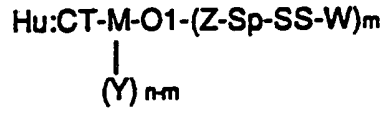
prepared from a compound of formula CH_3-SSS-W wherein CH_3-SSS-W is an antitumou antibiotic designated as LL-E33288α_1^{Br}, α_1^{I}, α_2^{Br}, α_3^{Br}, α_3^{I}, α_4^{Br}, β_1^{Br}, β_1^{I}, β_2^{Br}, β_2^{I}, γ_1^{Br}, γ_1^{I}, δ_1^{I}, the iodo or bromo pseudoaglycone, their dihydro or N-acyl counterparts, BBM-1675, FR-900405, FR-900406, PD 114759, PD 115028, CL-1577A, CL-1577B, CL-1577D, CL-1577E, CL 1724 or their N-acetyl counterparts comprising:

reacting CH_3-SSS-W with a compound of general formula Q-Sp-SH, wherein Sp is a straight or branched-chain divalent or trivalent (C_1-C_{18}) radical, divalent or trivalent aryl or heteroaryl radical,

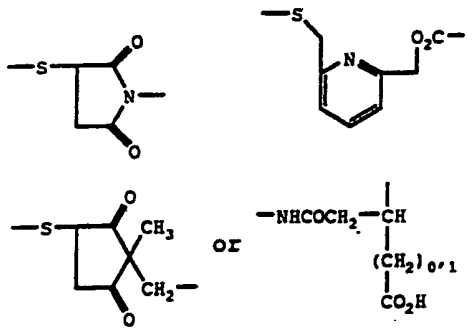
divalent or trivalent (C_3-C_{18}) cycloalkyl or heterocycloalkyl radical, divalent or trivalent aryl- or heteroaryl-alkyl (C_1-C_{18}) radical, divalent or trivalent cycloalkyl- or heterocycloalkyl-alkyl (C_1-C_{18}) radical or divalent or trivalent (C_2-C_{18}) unsaturated alkyl radical, wherein if Sp is a trivalent radical, it can be additionally substituted by amino, alkylamino, arylamino, heteroarylamino, carboxyl, lower alkoxy, hydroxy, thiol, or lower alkylthio groups; and Q is, or can be subsequently converted to, halogen, amino, alkylamino, carboxyl, carboxaldehyde, hydroxy, thiol, a-haloacetyloxy, lower alkyldicarboxyl, -$CONHNH_2$, -$NHCONHNH_2$, -$NHCSNHNH_2$, -ONH_2, -CON_3,



to produce an intermediate formula of the formula Q-Sp-SS-W, wherein Q, Sp, and W are as hereinbefore defined,
reacting Q-Sp-SS-W with a molecule of the formula Hu:CT-M-01-$(Y)_n$ wherein Hu:CT-M-O1 is a HAM according to Claims 1 to 17 and Y is a side-chain amino, carboxyl, or thiol group of a protein, an aldehyde derived from glycoprotein carbohydrate residues, or an amidoalkylthio group; and *n* is an integer of from 1 to 100, to produce a compound of the formula:

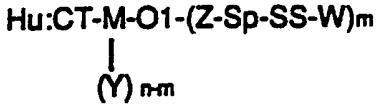


wherein Y, Sp, W, an *n* are as hereinbefore defined, and Z is formed from covalent reaction of the groups Q and Y directly or after subsequent reduction, and Z is -CONH-, -CONHN=CH-, -CONHNHCH$_2$-, -NHCSNHN=CH-, -NHCH$_2$-, -N=CH-, -CO$_2$-, -NHCH$_2$CO$_2$-, -SS-,



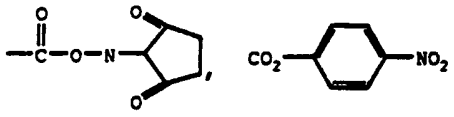
and *m* is 0.1 to 15.

27. A conjugate according to Claim 26 of the formula



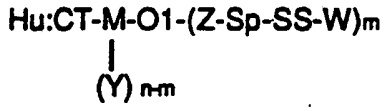
prepared from the class of antitumor antibiotics designated LL-E33288 (CH$_3$-SSS-W) comprising:

displacing the dithiomethyl moiety with a compound of formula Q-Sp-SH, wherein Sp is straight or branched-chain divalent or trivalent (C_2-C_{10}) radicals or divalent or trivalent aryl- or heteroarylalkyl (C_2-C_5) radicals, wherein if Sp is a trivalent radical, it can be additionally substituted by amino, heteroarylamino, hydroxy, or thiol groups; and Q is, or can be subsequently converted to, carboxyl, lower alkyldicarboxyl anhydride, -CONHNH$_2$, or

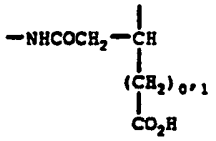


to produce an intermediate of general formula Q-Sp-SS-W, wherein Q, Sp, and W are as hereinbefore defined,

reacting Q-Sp-SS-W with a molecule of the formula Hu:CT-M-01-$(Y)_n$ wherein Y is a side-chain amino group on the antibody, or an aldehyde generated by oxidation of the carbohydrate groups of the antibody, and *n* is an integer of from 1 to 100, to produce a compound of the formula:



wherein Y, Sp, W, and *n* are as hereinbefore defined, and Z is formed from covalent reaction of the groups Q and Y directly or after subsequent reduction, and Z is -CONH-, -CONHN=CH-, -CONHNHCH$_2$-, or



and *m* is 0.1 to 15.

28. A conjugate according to Claim 27 wherein CH$_3$-SSS-W is the antitumour antibiotic designated LL-E33288γ_1^I.

29. A conjugate according to Claim 27 wherein CH$_3$-SSS-W is the antitumour antibiotic designated LL-E33288α_2^I.

30. A conjugate according to Claim 27 wherein CH$_3$-SSS-W is the antitumour antibiotic designated LL-E33288α_3^I.

31. A conjugate according to Claim 27 wherein CH_3-SSS-W is the antitumour antibiotic designated N-acetyl LL-E33288γ_1^I.

32. A conjugate according to Claim 27 wherein CH_3-SSS-W is the antitumour antibiotic designated iodo LL-E33288 pseudoaglycone.

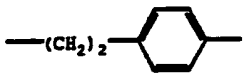
33. A conjugate according to Claim 27 wherein Q is the hydroxysuccinimide ester of a carboxyl group, Sp is -CH_2CH_2-, Y is -NH_2, Z is -CONH-, and *m* is 0.5 to 15.

34. A conjugate according to Claim 27 wherein Q is the hydroxysuccinimide ester of a carboxyl group, Sp is -$CH_2CH(CH_3)$-, Y is -NH_2, Z is -CONH-, and *m* is 0.5 to 15.

35. A conjugate according to Claim 27 wherein Q is the 4-nitrophenyl ester of a carboxyl group, Sp is -CH_2CH_2-, Y is -NH_2, Z is -CONH-, and *m* is 0.5 to 15.

36. A conjugate according to Claim 27 wherein Q is the hydroxysuccinimide ester of a carboxyl group, Sp is -$CH_2C(CH_3)_2$-, Y is -NH_2, Z is -CONH-, and *m* is 0.5 to 15.

37. A conjugate according to Claim 27 wherein Q is the hydroxysuccinimide ester of a carboxyl group, Sp is



Y is -NH_2, Z is -CONH-, and *m* is 0.5 to 15.

38. A conjugate according to Claim 27 wherein Q is -$CONHNH_2$, Sp is -CH_2CH_2-, Y is -CHO, Z is -CONHN=CH-, and *m* is 0.1 to 10.

39. A conjugate according to Claim 27 wherein Q is -$CONHNH_2$, Sp is -CH_2CH_2-, Y is -CHO, Z is -$CONHNHCH_2$-, and *m* is 0.1 to 10.

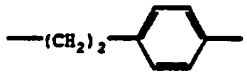
40. A conjugate according to Claim 27 wherein Q is -$CONHNH_2$, Sp is -$CH_2CH(CH_3)$-, Y is -CHO, Z is -CONHN=CH-, and *m* is 0.1 to 10.

41. A conjugate according to Claim 27 wherein Q is -$CONHNH_2$, Sp is -$CH_2CH(CH_3)$-, Y is -CHO, Z is -$CONHNHCH_2$-, and *m* is 0.1 to 10.

42. A conjugate according to Claim 27 wherein Q is -$CONHNH_2$, Sp is -$CH_2C(CH_3)_2$-, Y is -CHO, Z is -CONHN=CH-, and *m* is 0.1 to 10.

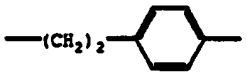
43. A conjugate according to Claim 27 wherein Q is -$CONHNH_2$, Sp is -$CH_2C(CH_3)_2$-, Y is -CHO, Z is -$CONHNHCH_2$-, and *m* is 0.1 to 10.

44. A conjugate according to Claim 27 wherein Q is -$CONHNH_2$, Sp is



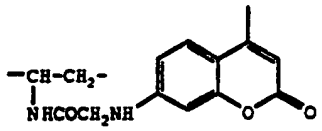
Y is -CHO, Z is -CONHN=CH-, and *m* is 0.1 to 10.

45. A conjugate according to Claim 27 wherein Q is -$CONHNH_2$, Sp is



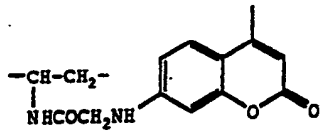
Y is -CHO, Z is -$CONHNHCH_2$-, and *m* is 0.1 to 10.

46. A conjugate according to Claim 27 wherein Q is -$CONHNH_2$, Sp is



Y is -CHO, Z is -CONHN=CH-, and *m* is 0.1 to 10.

47. A conjugate according to Claim 27 wherein Q is -$CONHNH_2$, Sp is



Y is -CHO, Z is -$CONHNHCH_2$-, and *m* is 0.1 to 10.

48. A conjugate according to Claim 38 wherein CH_3-SSS-W is LL-E33288γ_1^I, Q is -$CONHNH_2$, Sp is -CH_2CH_2-, Y is -CHO, Z is -CONHN=CH-, and *m* is 0.1 to 10.

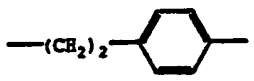
49. A conjugate according to Claim 39 wherein CH_3-SSS-W is LL-E33288α_3^I, Q is -$CONHNH_2$, Sp is -CH_2CH_2-, Y is -CHO, Z is -$CONHNHCH_2$-, and *m* is 0.1 to 10.

50. A conjugate according to Claim 33 wherein CH_3-SSS-W is N-acetyl LL-E33288γ_1^I, Q is hydroxysuccinimidocarbonyl, Sp is -CH_2CH_2-, Y is -CHO, Z is -CONH-, and *m* is 0.5 to 15.

51. A conjugate according to Claim 34 wherein CH_3-SSS-W is N-acetyl LL-E33288γ_1^I, Q is hydroxysuccinimidocarbonyl, Sp is -$CH_2CH(CH_3)$-, Y is -NH_2, Z is -CONH-, and *m* is 0.5 to 15.

52. A conjugate according to Claim 36 wherein CH_3-SSS-W is N-acetyl LL-E33288γ_1^I Y, Q is hydroxysuccinimidocarbonyl, Sp is -$CH_2C(CH_3)_2$-, Y is -NH_2, Z is -CONH-, and *m* is 0.5 to 15.

53. A conjugate according to Claim 44 wherein CH_3-SSS-W is N-acetyl LL-E33288γ_1^I, Q is -$CONHNH_2$, Sp is



Y is -CHO, Z is -CONNH=CH-, and *m* is 0.1 to 10.

54. A pharmaceutical composition containing a HAM according to Claim 1 together with a pharmaceutically acceptable excipient., diluent or carrier.

55. A pharmaceutical composition according to Claim 54 wherein the HAM is conjugated to an effector or reporter molecule.

56. A method for treating a carcinoma in a human subject which comprises administering a pharmaceutically effective amount of a HAM according to Claim 1.

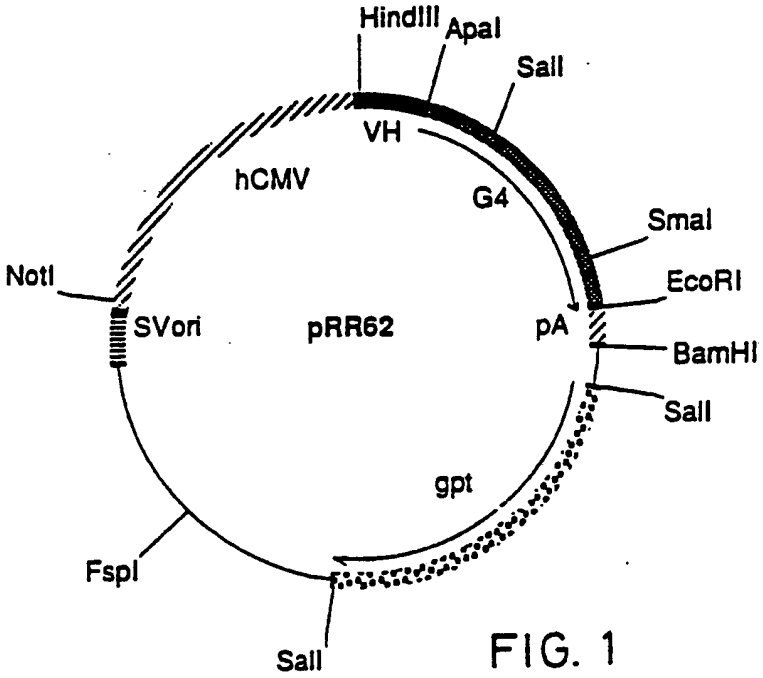

HindIII
ApaI
SalI
VH
hCMV
G4
SmaI
EcoRI
NotI
SVori
pRR62
pA
BamHI
SalI
gpt
FspI
SalI

FIG. 1

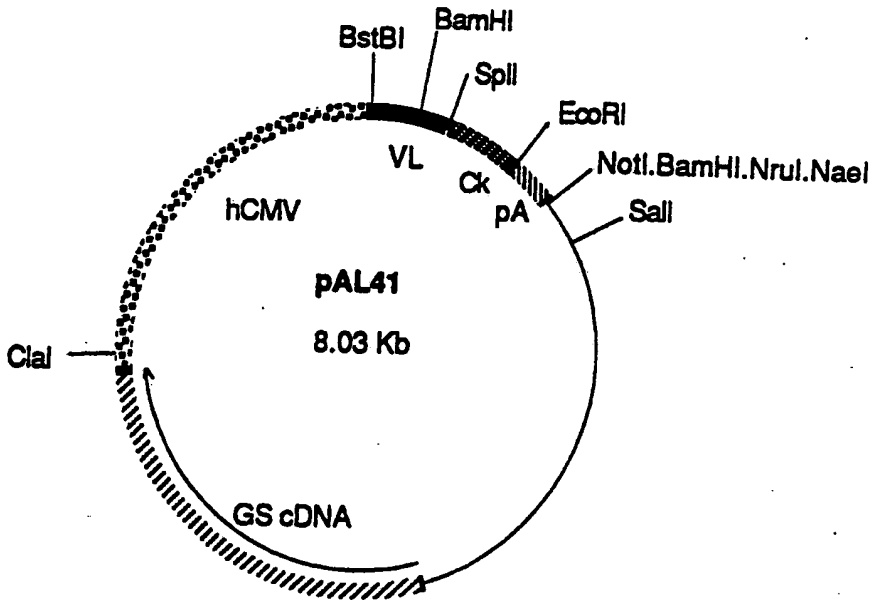

BstBI
BamHI
SpII
EcoRI
VL
Ck
NotI.BamHI.NruI.NaeI
hCMV
pA
SalI
pAL41
8.03 Kb
ClaI
GS cDNA

FIG. 2

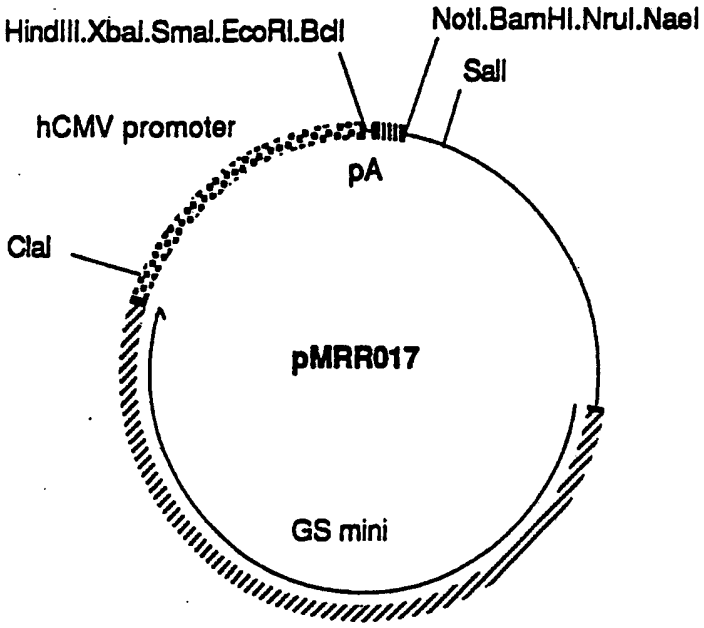

HindIII.Xbal.Smal.EcoRI.BclI
NotI.BamHI.NruI.NaeI
SalI
hCMV promoter
pA
ClaI
pMRR017
GS mini

FIG. 3

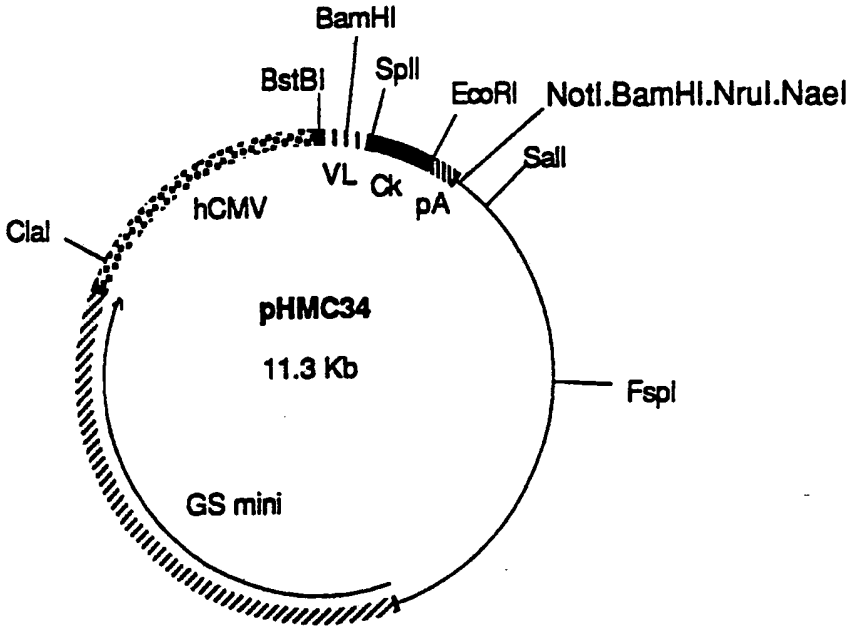

BamHI
BstBI
SpII
EcoRI
NotI.BamHI.NruI.NaeI
SalI
VL
Ck
pA
hCMV
ClaI
pHMC34
11.3 Kb
FspI
GS mini

FIG. 4

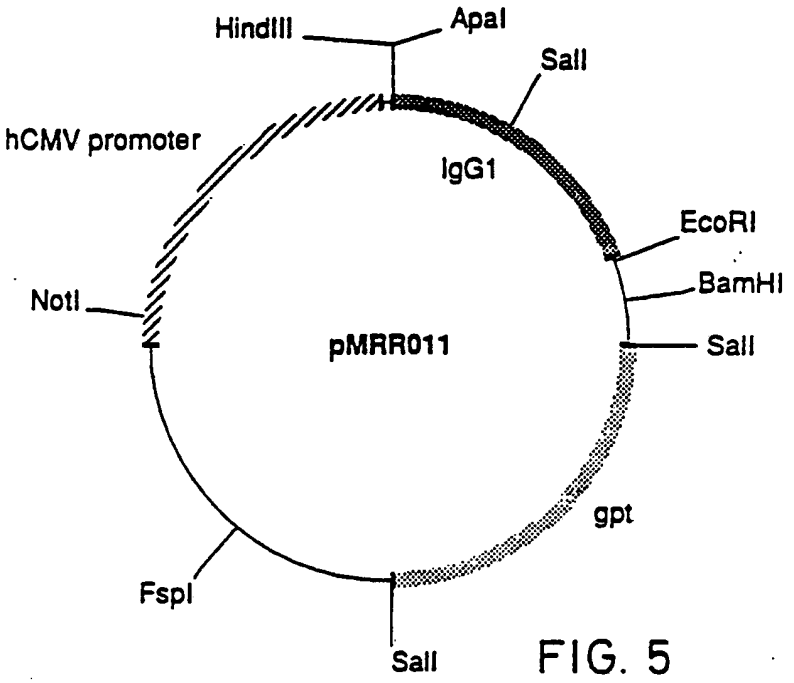

HindIII
ApaI
SalI
hCMV promoter
IgG1
EcoRI
BamHI
NotI
pMRR011
SalI
gpt
FspI
SalI

FIG. 5

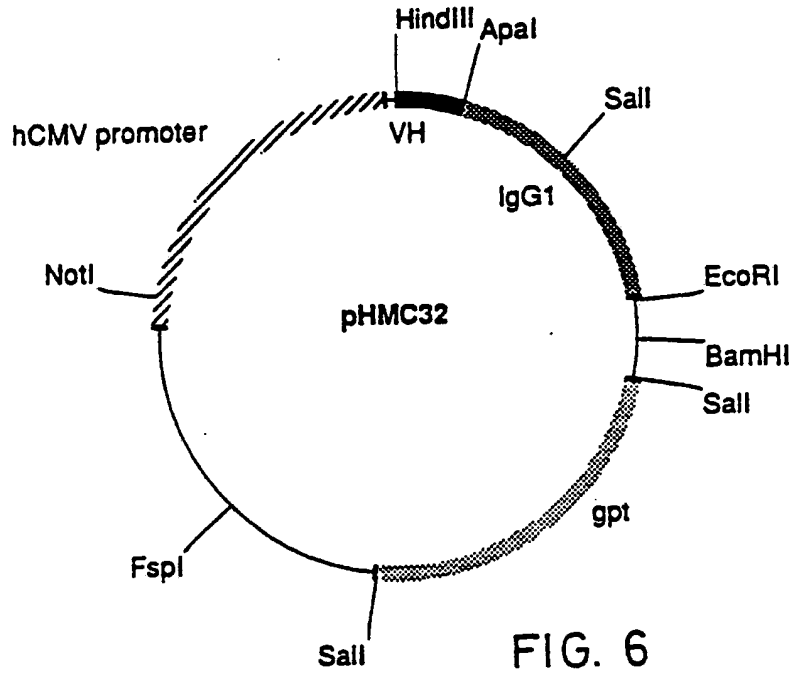

HindIII
ApaI
SalI
hCMV promoter
VH
IgG1
NotI
EcoRI
pHMC32
BamHI
SalI
gpt
FspI
SalI

FIG. 6

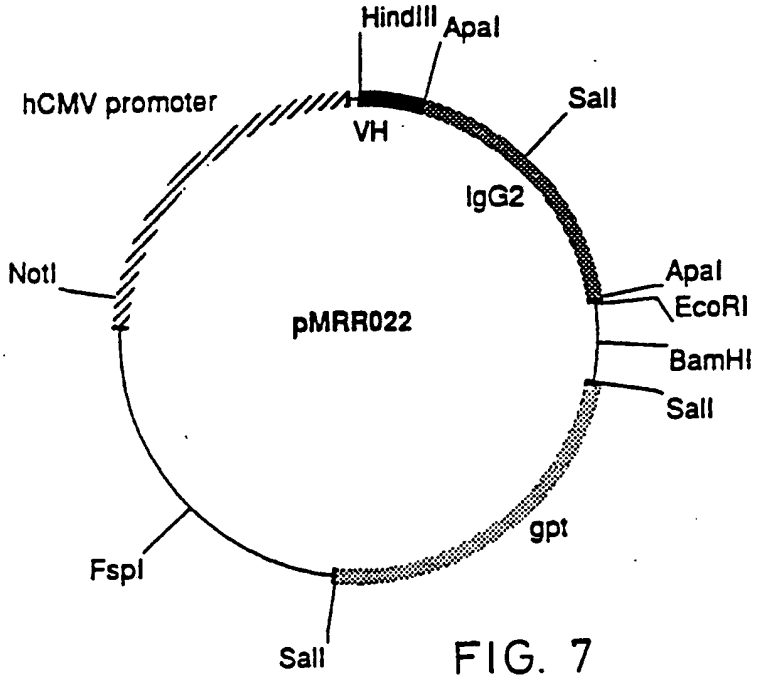

HindIII
ApaI
hCMV promoter
VH
SalI
IgG2
ApaI
EcoRI
BamHI
SalI
NotI
pMRR022
gpt
FspI
SalI

FIG. 7

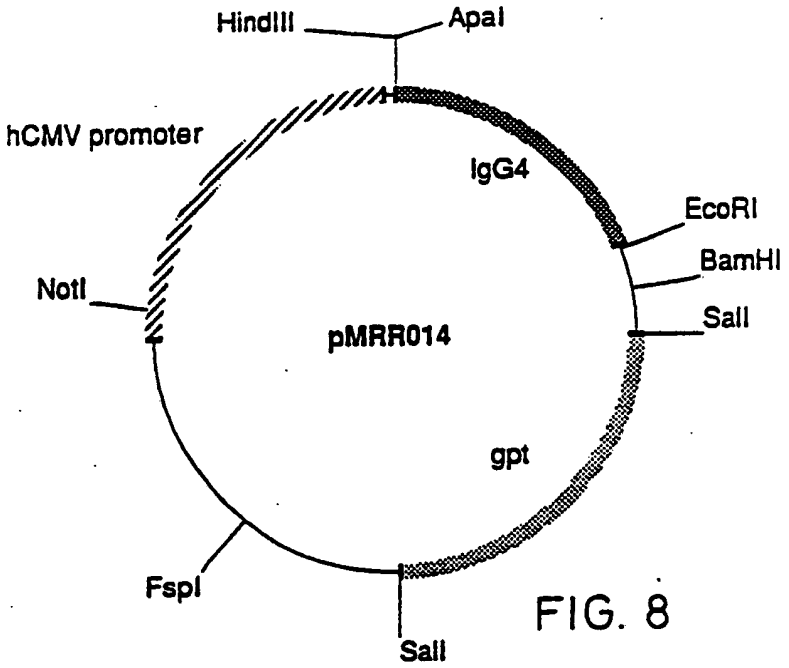

HindIII
ApaI
hCMV promoter
IgG4
EcoRI
BamHI
SalI
NotI
pMRR014
gpt
FspI
SalI

FIG. 8

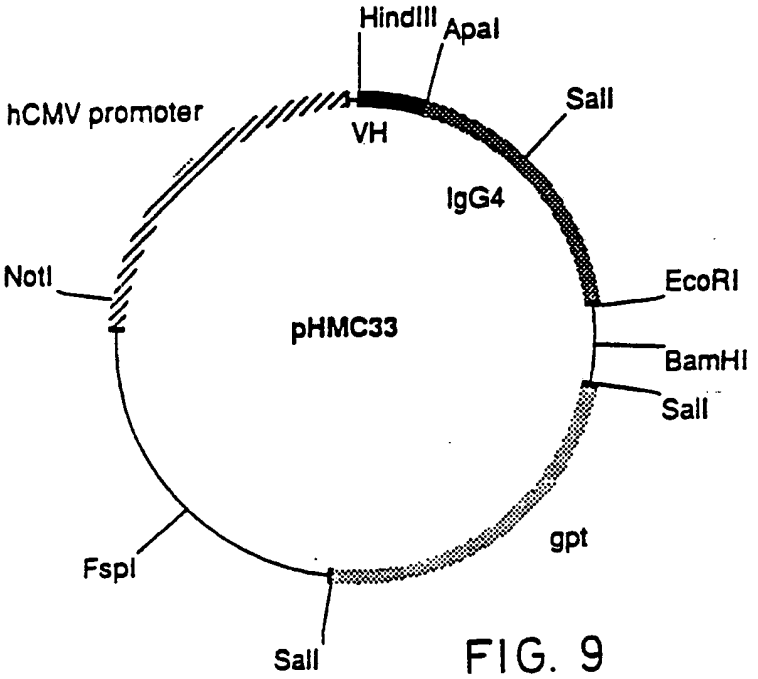

HindIII
ApaI
SalI
hCMV promoter
VH
IgG4
NotI
EcoRI
pHMC33
BamHI
SalI
gpt
FspI
SalI

FIG. 9

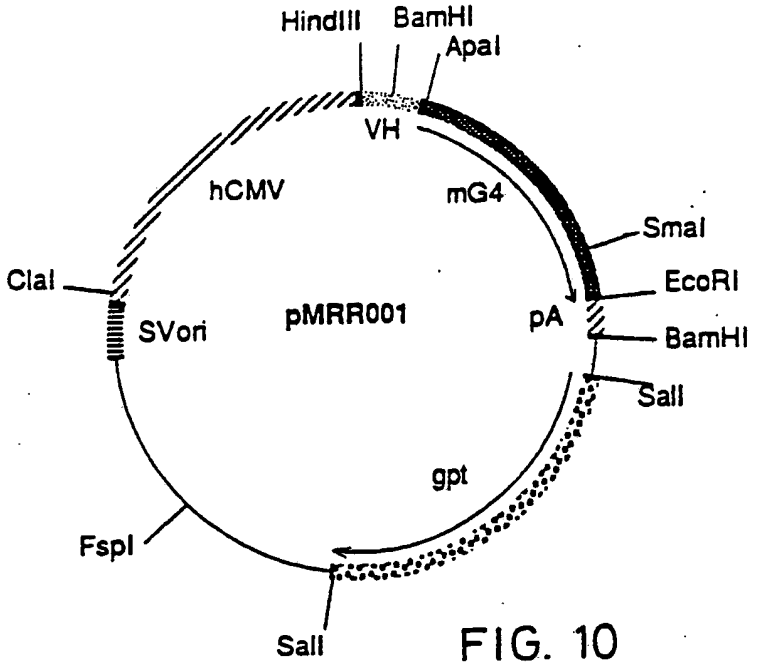

HindIII
BamHI
ApaI
VH
hCMV
mG4
SmaI
ClaI
EcoRI
SVori
pMRR001
pA
BamHI
SalI
gpt
FspI
SalI

FIG. 10

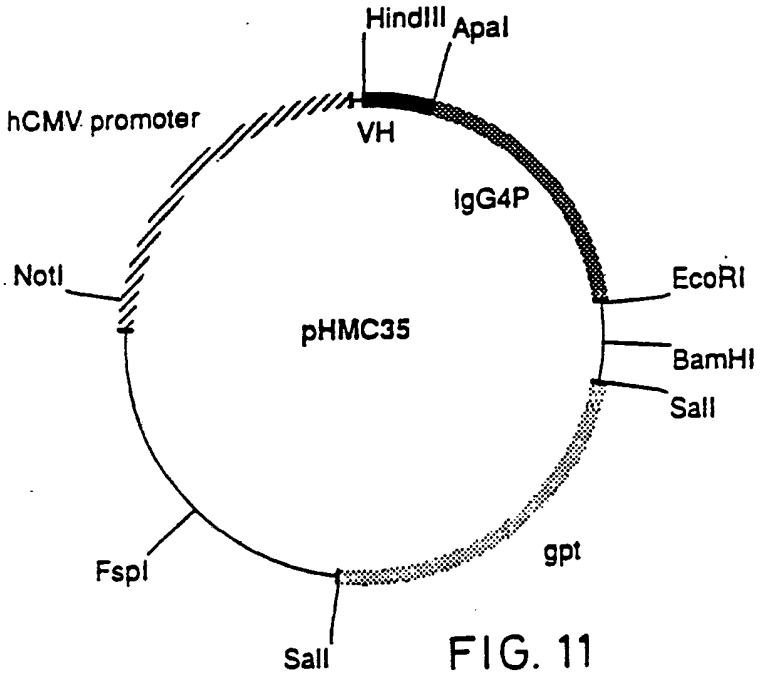

HindIII
ApaI
hCMV promoter
VH
IgG4P
NotI
EcoRI
pHMC35
BamHI
SalI
FspI
gpt
SalI

FIG. 11

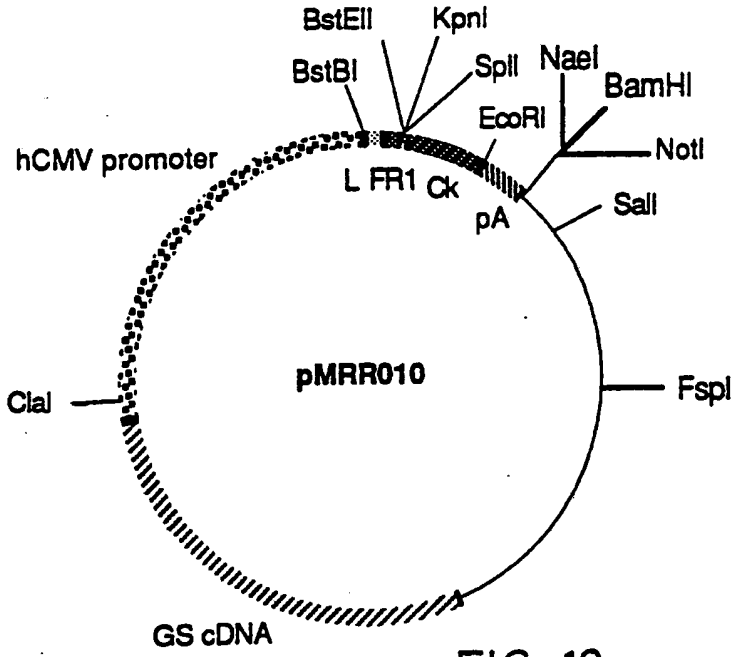

BstEII
KpnI
BstBI
SpII
NaeI
BamHI
EcoRI
NotI
hCMV promoter
L FR1
Ck
pA
SalI
pMRR010
FspI
ClaI
GS cDNA

FIG. 19

FIG. 13

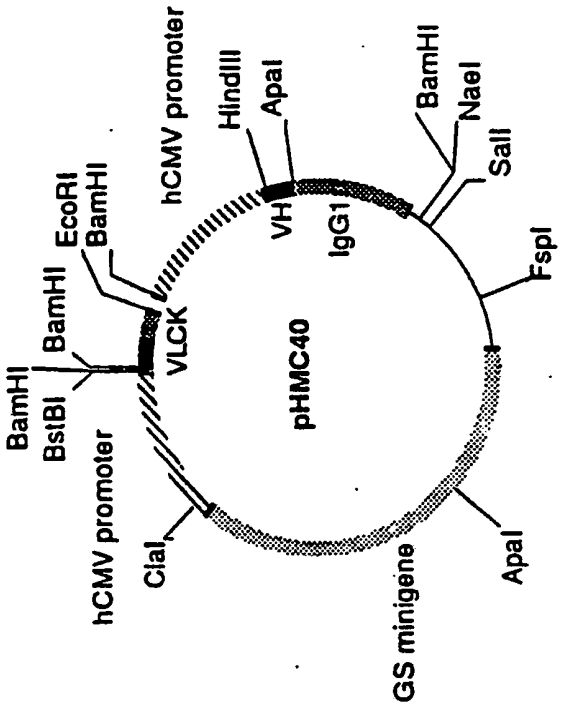

hCMV promoter
HindIII
ApaI
BamHI
NaeI
SalI
FspI
EcoRI
BamHI
VH
IgG1
BamHI
BamHI
BstBI
VLCK
pHMC40
hCMV promoter
ClaI
ApaI
GS minigene

FIG. 12

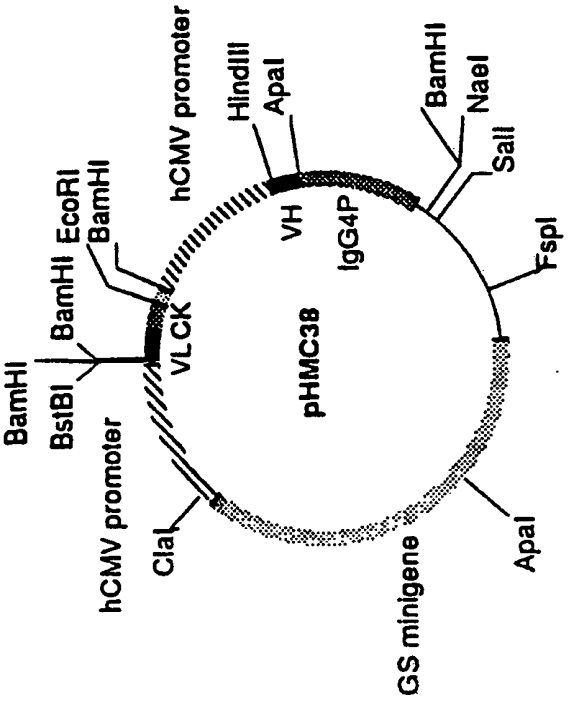

hCMV promoter
HindIII
ApaI
BamHI
NaeI
SalI
FspI
EcoRI
BamHI
VH
IgG4P
BamHI
BamHI
BstBI
VLCK
pHMC38
hCMV promoter
ClaI
ApaI
GS minigene

FIG. 15

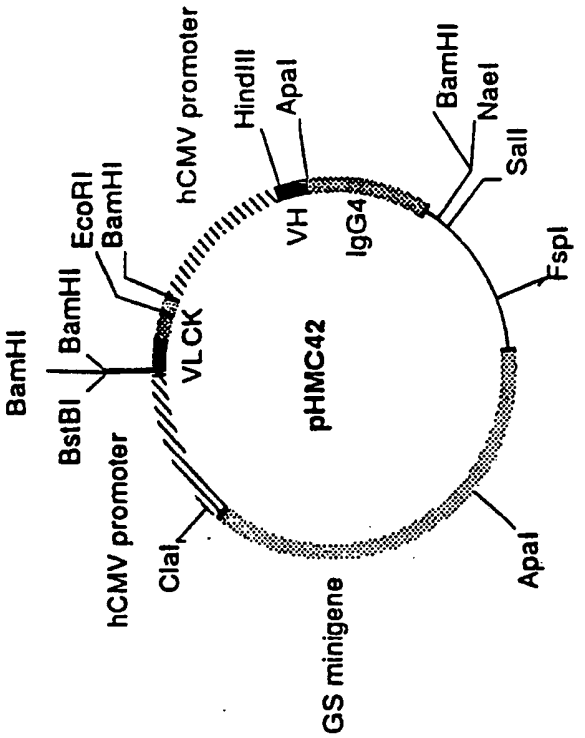
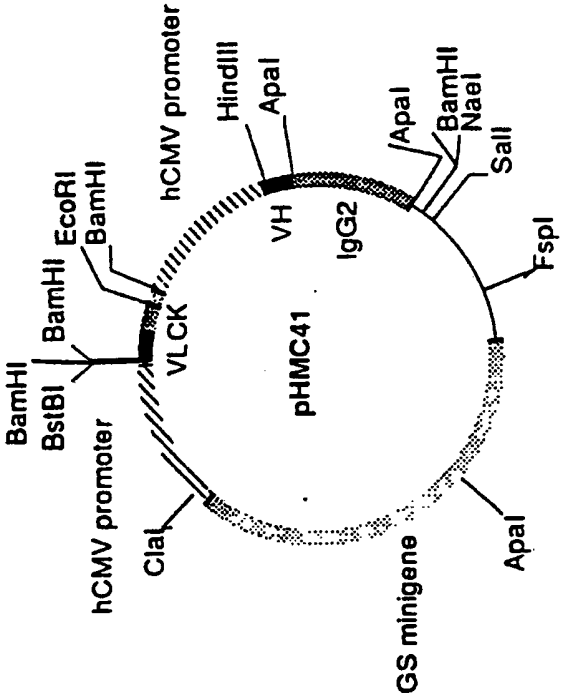

pHMC42
hCMV promoter
HindIII
ApaI
BamHI
NaeI
SalI
FspI
EcoRI
BamHI
VH
IgG4
BamHI
BamHI
BstBI
VLCK
hCMV promoter
ClaI
ApaI
GS minigene

FIG. 14


pHMC41
hCMV promoter
HindIII
ApaI
ApaI
BamHI
NaeI
SalI
FspI
EcoRI
BamHI
VH
IgG2
BamHI
BamHI
BstBI
VLCK
hCMV promoter
ClaI
ApaI
GS minigene

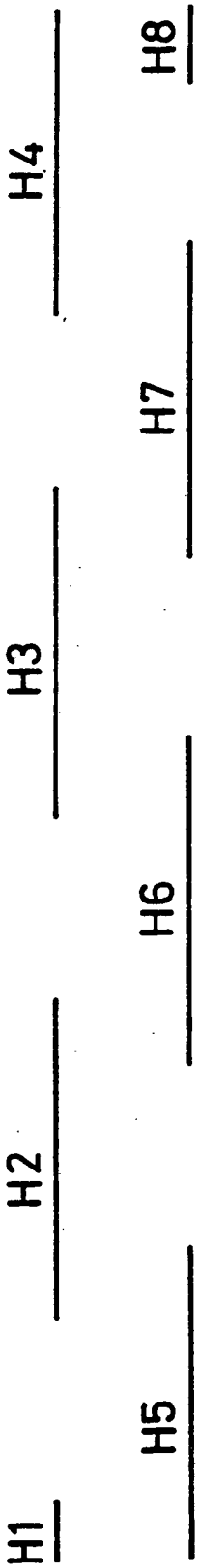


FIG. 16

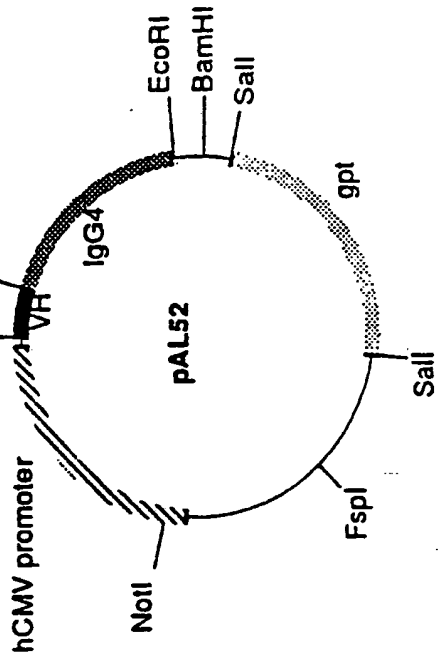


FIG. 18

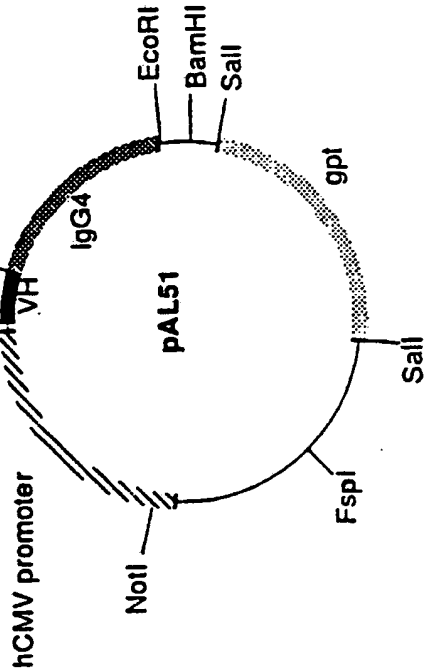


FIG. 17

FIG. 21

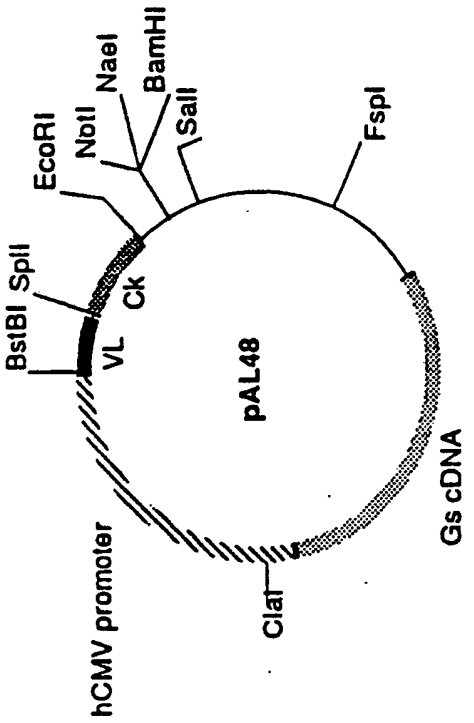
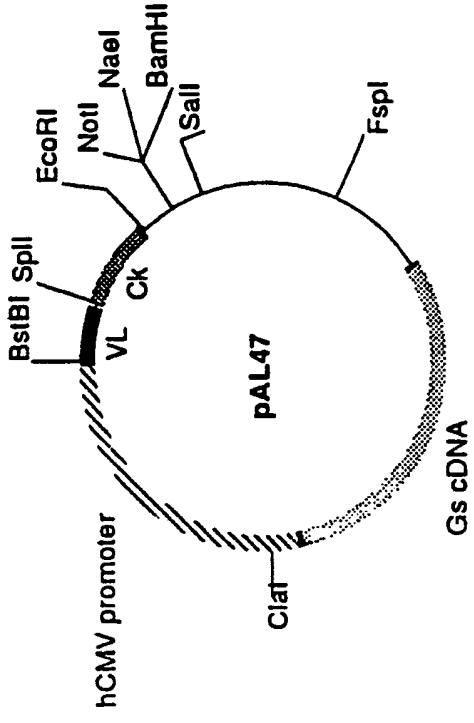

pAL48
hCMV promoter
BstBI
SpII
VL
Ck
EcoRI
NotI
NaeI
BamHI
SalI
FspI
ClaI
Gs cDNA

FIG. 20


pAL47
hCMV promoter
BstBI
SpII
VL
Ck
EcoRI
NotI
NaeI
BamHI
SalI
FspI
ClaI
Gs cDNA

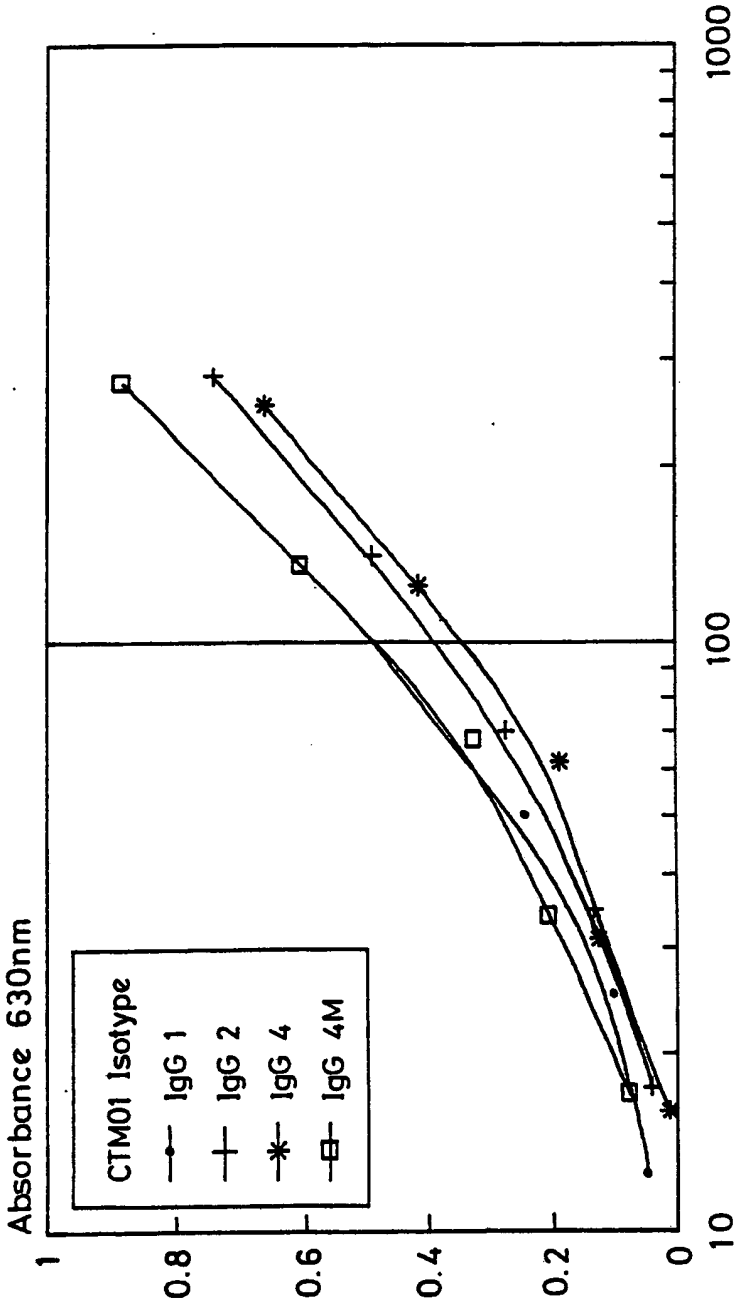

Absorbance 630nm
1
0.8
0.6
0.4
0.2
0
10
100
1000
Antibody Conc ng/ml
CTM01 Isotype
IgG 1
IgG 2
IgG 4
IgG 4M

FIG. 22

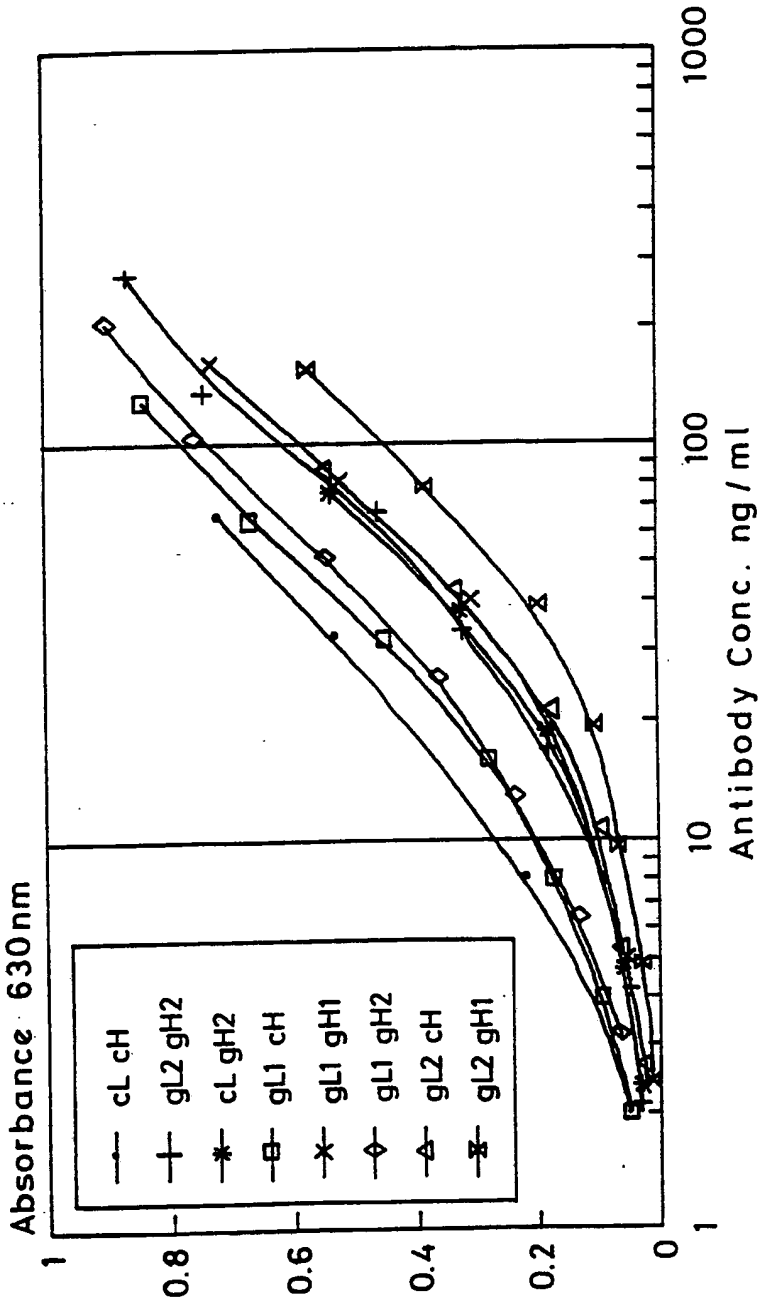
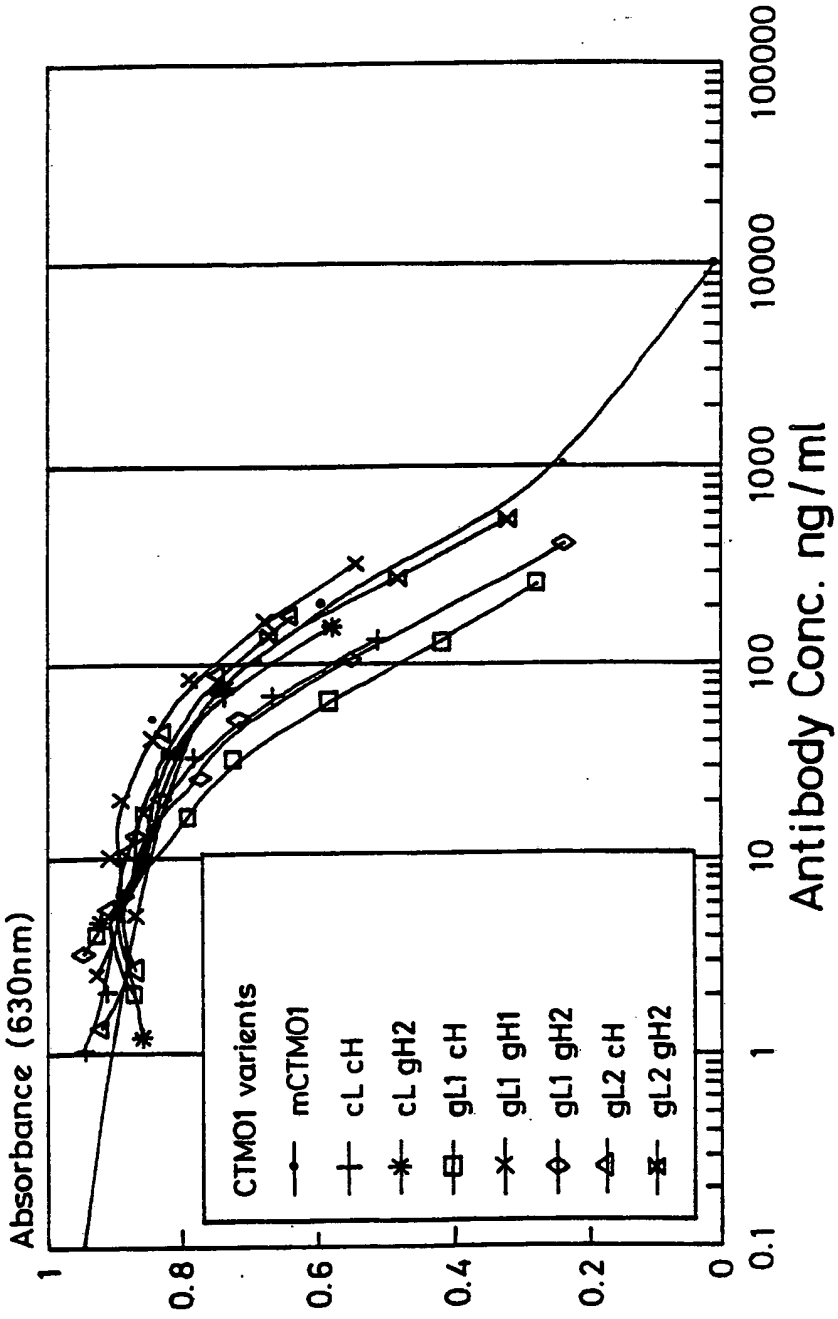

Absorbance 630nm
1
0.8
0.6
0.4
0.2
0
1
10
100
1000
Antibody Conc. ng/ml
cL cH
gL2 gH2
cL gH2
gL1 cH
gL1 gH1
gL1 gH2
gL2 cH
gL2 gH1

FIG. 23

FIG. 24


Absorbance (630nm)
1
0.8
0.6
0.4
0.2
0
0.1
1
10
100
1000
10000
100000
Antibody Conc. ng/ml
CTMO1 variants
mCTMO1
cL cH
cL gH2
gL1 cH
gL1 gH1
gL1 gH2
gL2 cH
gL2 gH2

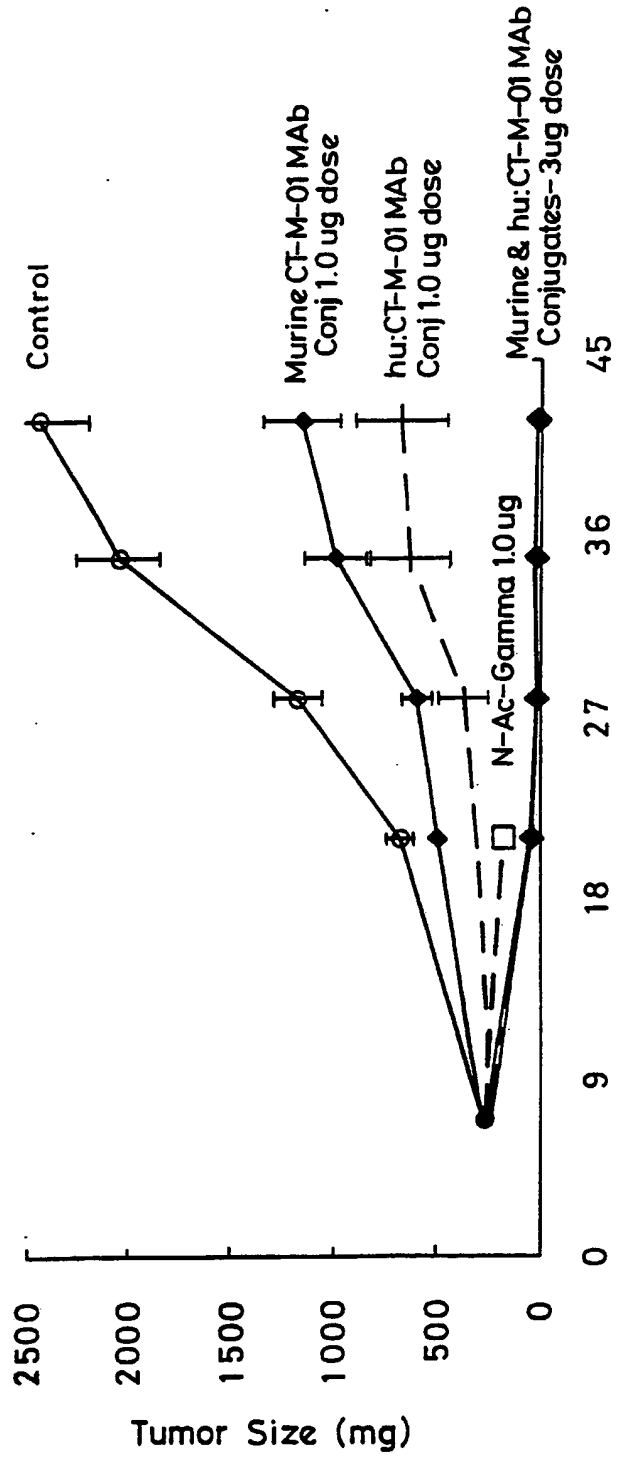

Control
Murine CT-M-01 MAb
Conj 1.0 ug dose
hu:CT-M-01 MAb
Conj 1.0 ug dose
N-Ac-Gamma 1.0 ug
Murine & hu:CT-M-01 MAb
Conjugates-3ug dose
2500
2000
1500
1000
500
0
Tumor Size (mg)
0
9
18
27
36
45
Days Post Implantation

FIG. 25

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 92/01759

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12P21/08; C12N15/13; A61K47/48; A61K39/395

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	A61K ; C12P ; C12N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
Y	EP,A,0 392 384 (AMERICAN CYANAMID COMPANY) 17 October 1990 cited in the application see the whole document, especially page 10 lines 22-35 ---	1-11, 15-56
Y	WO,A,9 109 967 (CELLTECH LIMITED) 11 July 1991 cited in the application see the whole document ---	1-11, 15-56
A	EP,A,0 208 615 (IRE-CELLTARG S.A.) 14 January 1987 see the whole document -----	1-56

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

2

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
25 NOVEMBER 1992	05. 12. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	FERNANDEZ Y BRA F.

national application No.

PCT/GB92/01759

INTERNATIONAL SEARCH REPORT

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 56 is directed to a method of treatment of the human body the search has been carried out and based on the alleged effects of the composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9201759 SA 64804

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 25/11/92

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0392384	17-10-90	US-A-	5053394	01-10-91
		AU-A-	5324190	01-11-90
		CA-A-	2014486	14-10-90
		CN-A-	1046333	24-10-90
		JP-A-	2292294	03-12-90
		CA-A-	2014459	14-10-90
WO-A-9109967	11-07-91	AU-A-	6974091	24-07-91
		AU-A-	7033091	24-07-91
		AU-A-	7048691	24-07-91
		EP-A-	0460167	11-12-91
		EP-A-	0460171	11-12-91
		EP-A-	0460178	11-12-91
		WO-A-	9109966	11-07-91
		WO-A-	9109968	11-07-91
		GB-A-	2246781	12-02-92
		GB-A-	2246570	05-02-92
		JP-T-	4505398	24-09-92
EP-A-0208615	14-01-87	FR-A-	2584293	09-01-87
		DE-A-	3684050	09-04-92
		JP-A-	62070320	31-03-87